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

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Abstract

The aim of our study was to determine whether or not the tyrosine kinase receptor, HER2 (also known as ErbB2/Her2/*neu*), is overexpressed in human osteosarcomas (OS). We studied 15 biopsy and 18 resection specimens at the mRNA and protein levels. HER2 status in the OS specimens was assessed by immunohistochemistry (IHC) and quantitative Real-Time Polymerase chain reaction (PCR). In moderately immunopositive cases fluorescent *in situ* hybridisation (FISH) analysis was used in order to identify any possible gene amplification. 27 samples were evaluable for IHC and only 1 case showed a moderately positive membrane staining. The remaining samples showed no staining or focal cytoplasmic 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 as a negative control. In conclusion, this study shows that HER2 mRNA or membranous HER2 protein overexpression is absent in human OS. 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 is 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 OS.

Keywords: Osteosarcoma; Bone sarcomas; Bone tumours; Cancer therapy; Investigational treatment; Her-2 or HER2; Epidermal growth factor receptor; Herceptin; Prognosis

1. Introduction

Osteosarcoma (OS) is the most common primary bone tumour, with an incidence (on average) of 6.5 patients per 10^6 children per year 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 tumour volume, older age at presentation, axial site of the tumour, histological subtype of OS and histological response on preoperative treatment have all been associated with a poor outcome [2–4]. However, apart from metastatic disease and an axial site, that occurs 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 the time of diagnosis [2].

With the recent progress in the understanding of the molecular biology of cancer, the cell surface receptor,

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HER2 (also called $p185^{HER2}$), has been suggested to be predictive for survival [5–8].

The *HER-2* gene (also known as ERBB2 or the *neu* gene), located on chromosome 17q21 [9], encodes for a 185 kD transmembrane receptor [10] and belongs to the epidermal growth factor (EGF) tyrosine kinase receptor superfamily [11]. *HER-2* is an oncogene and *HER-2* overexpression *in vitro* [12] and in human cancers, particularly in 25–30% of breast cancer patients, has been associated with disease behaviour [13].

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 [14]. This oncogenic effect can be reverted by the use of monoclonal antibodies, directed against the HER2 protein [15]. Furthermore, both *in vitro* and clinical studies have reported increased response rates to chemotherapeutic drugs when these are combined with anti-HER2 antibodies [16]. Based on these results, and reports that *HER-2* is overexpressed in OS, phase II trials have begun to study the efficacy of Herceptin® (trastuzumab), the commercial designation of humanised HER2 monoclonal antibody, in patients with relapsed or refractory OS [6,17] (www.cancer.gov/clinical_trials: MSKCC-99097/NCI-T98-0083 and COG-AOST0121).

Four studies have suggested that *HER-2* is overexpressed in OS. However, they report different correlations between *HER-2* overexpression and prognosis [5–7,18]. Furthermore, other investigators have not been able to confirm their conclusions [17,19,20].

Thus, in order to clarify these conflicting results and to investigate whether trastuzumab is a suitable therapy for OS, we studied the expression of the *HER-2* gene by assessing gene amplification, mRNA- and protein expressions of HER2 in 30 patients.

2. Patients and methods

2.1. Patients

All patients presented to the Department of Orthopedic Surgery of the Leiden University Medical Center with newly-diagnosed high-grade osteosarcoma (OS) of the limbs ($n = 32$) and the os ileum ($n = 1$) from 1991 to 1999 (Table 1). Diagnosis was made using routine haematoxylin-eosin (HE) staining in 15 pre-treatment Jamshidi 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 European Osteosarcoma Intergroup (EOI) studies, such as European Organisation for Research and Treatment of Cancer (EORTC) 80861 [21] and 80871 studies [22], the EOI phase II study of intensive chemotherapy with granulocyte-colony stimulating factor (G-CSF) [23] or the recently closed EORTC 80931 trial. Patients who did not enter a trial (either refused or were not eligible) were offered short intensive courses of chemotherapy, as described by Bramwell and colleagues in Ref. [24]. One patient did not receive chemotherapy because of advanced age. Another patient was treated with doxorubicin only, as palliative therapy. Histological response after preoperative 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% of 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.

2.2. 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 instructions. For isolation of mRNA, only tissue containing more than 50% of tumour cells was selected.

2.3. Quantification of HER2 transcripts with TaqMan Real-Time PCR

HER2 expression was determined by quantitative real-time polymerase chain reaction (qPCR) using cDNA synthesised 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) and 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 a 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 in Ref. [25]. Primers for HER2 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).

Table 1
Patient's clinical data and results of our study

Patient number	Age at Dx	Sample	Localisation of tumour	Histological subtype	Chemotherapy 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 amplification
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 tumour	
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 tumour	
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 tumour	
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	Pe	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		

B, biopsy; R, resection; Fe, femur; Ti, tibia; Hu, humerus; Cla, clavicle; Pe, pelvis; Co, high-grade conventional; Ob, osteoblastic; Te, teleangiectatic; MFH, MFH-like; PR, poor response; GR, good response; Surv, survived; DOD, died of disease; NA, not available; Dx, diagnosis; IHC, immunohistochemistry; FISH, fluorescent *in situ* hybridisation; MFH, malignant fibrous histiocytoma-like.

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 overexpression of HER2-mRNA [26] was used as a 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 [26] and was used as a negative control for HER2 overexpression.

2.4. Immunohistochemical analysis

Paraffin-embedded, formalin-fixed tissue samples were used for the immunohistochemical (IHC) analyses. 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 the 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 the DAKO HERCEPTEST®.

2.5. FISH for HER2 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 HER2 gene amplification, according to the

manufacturer's instructions. Using a fluorescent microscope, the HER2 copy number and the centromere chromosome 17 copy numbers were counted in the tumour cells.

3. Results

3.1. Patients

Patients' clinical characteristics and outcome are listed in Table 1. Biopsy samples of 15 patients (group A) were studied. All 15 samples were from primary tumours, three of which later relapsed (nos. 5, 12 and 15). Samples of group B consisted of 12 post-chemotherapy resections of OS 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 the primary diagnosis were included in the upper panel (nos. 5, 12 and 15, respectively). The mean age of patients in group A was lower (22 years, range 7–48 years) than those in group B (mean 37 years, range 14–82 years). The localisation of the OS was similar in both groups, mainly in the femur (in 80 and 72% in groups A and B, respectively). Other sites were the tibia in 2 cases in each group and 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 teleangiectatic, and one had a malignant fibrous histiocytoma (MFH)-like subtype (see Table 1).

3.2. HER2 mRNA expression

HER2 mRNA expression was assessed by Real-Time PCR, using RNA from the OS specimens. The mean absolute value for HER2 mRNA expression in group A was 0.017 (range 0.003–0.034) and 0.025 (range 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 had 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 that is observed in normal breast tissues. Even the highest value observed in group B (0.105) can be regarded as not being overexpressed particularly as no protein expression was seen in this sample.

3.3. Immunohistochemistry

Three of the 33 samples were lost after immunostaining during the retrieval procedures. In an additional three samples, no vital tumour was left in the histological sections. IHC was not repeated in these samples. Nearly all of the samples showed no HER2 plasma membrane-associated staining. An example of an OS sample that stained negative by IHC is shown in Fig. 1(a). Fig. 1(b) shows a positive membrane staining in a control breast cancer sample with proven *HER-2* gene amplification. This represents a 3+ score. Only one OS sample (patient no. 4) showed moderately positive immunostaining of the membrane, which was scored as 2+ (Fig. 1(c)).

Focal cytoplasmic IHC positivity was seen in two other samples, but as discussed previously, this was not considered as positive for HER2 overexpression.

3.4. *Fluorescent* in situ *hybridisation (FISH)*

FISH was performed in the OS sample with 2+ positive membrane staining and did not show any *HER-2* gene amplification.

4. Discussion

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

Usually, the HER2 protein is overexpressed as result of *HER-2* gene amplification and concomitant elevated mRNA expression [27]. Nevertheless, protein overexpression has been reported in the absence of gene amplification [13,28–30]. 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 [31,32].

HER2 status in OS has been investigated in eight other clinical studies [5–8,17–20] (Table 2). In five of these studies, HER2 overexpression was reported to occur in 42–63% of the patients with primary non-metastatic OS, and in 10–58% of the cases that had pulmonary metastases at diagnosis [6,7] or had relapsed [5,8,18]. Three studies correlated HER2 overexpression with a poor response to pre-operative chemotherapy and a poor outcome [5,6,8]. Remarkably, two other studies conclude that HER2 overexpression predicts a better survival and is less frequent in metastatic disease [7,18].

In the four remaining studies, including ours, no HER2 overexpression could be demonstrated [17,19,20]. These inconsistent findings regarding HER2 status and its significance in OS raise questions about the reliability

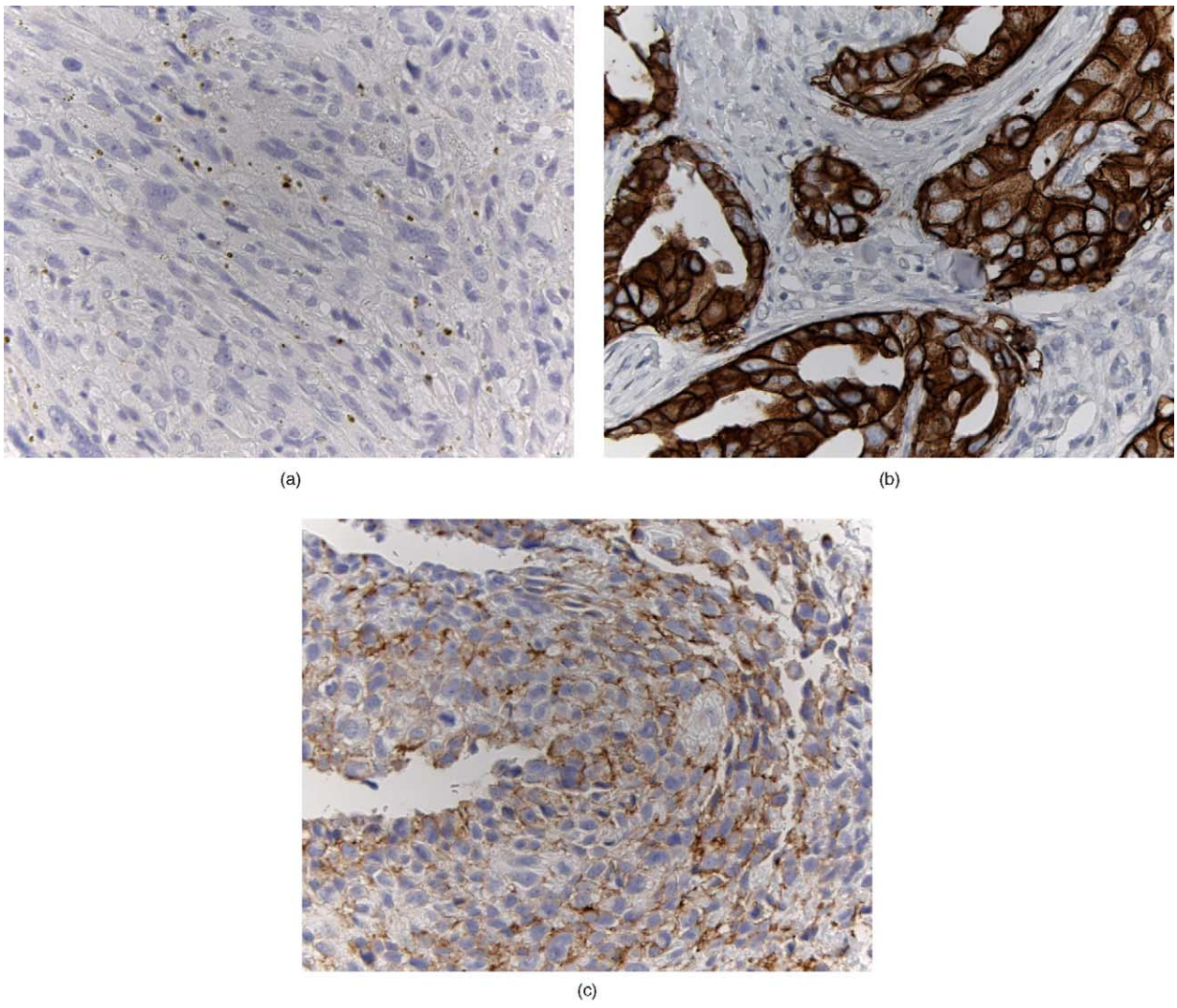

Fig. 1. Immunohistochemical staining for HER-2 protein expression: (a) osteosarcoma negative for HER-2; (b) breast cancer sample positive for membrane-associated HER-2 expression (score 3+); (c) osteosarcoma from patient no. 4 with moderate positive expression of HER-2 (score 2+).

of some of the studies and may be explained by methodological differences.

The HER2 status in the published studies has been assessed mainly by IHC. These studies differed considerably in their use of antibody and quality controls, scoring systems, interpretation of positivity of the sample and validation of the IHC result. In breast cancer, HER2 testing and standardisation of the method used 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 tests have been reported, due to 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 OS studies [5–8,18]. Interpretation of the stained samples may have a high interobserver variability and a low rate of reproducibility [36,37]. This particularly occurs when the staining is heterogeneous, weakly-positive, in non-malignant cells, cytoplasmic staining or when retraction artifacts occur [38]. Cytoplasmic immunostaining is considered to be an IHC artefact [20,39], and only complete membrane staining should be counted as positive when interpreting the results [38]. Only one of the five OS studies that scored membrane staining specifically, reported any positive results [6]. However, a poorly characterised antibody, 5B5, was used in this particular study and this antibody has not been used in any other studies.

Most of the OS studies included the mandatory positive and negative controls for IHC, usually a patient sample with and without known HER2 overexpression. However, our series was the only one to use positive and negative cell lines as controls.

Table 2
Overview of clinical HER2 studies in osteosarcoma

Author [Ref.]	Number of patient samples		Immunohistochemistry			Other assays	% HER2+ samples		HER2-positive IHC samples related to		
			Antibody	Scoring system	Control		IHC+	Other assay	PR (%)	EFS (%)	OAS (%)
Onda [5]	26	26	CB11 CBE1	Qualitative (M)	–	IB, SB, SSCP	42	0	67	–	14 HER+ 84 HER–
Gorlick [6]	53	53	5B5 Herceptest®	Semi-quantitative (M)	+	–	45	ND	57	47 HER+ 79 HER–	–
Akatsuka [7]	81	81	CB11	Semi-quantitative (M and/or Cy)	+	–	63	ND	58	72 HER+ 46 HER–	82 HER+ 57 HER–
Akatsuka [18]	19	38	CB11	Semi-quantitative (M and/or Cy)	–	–	PT 50 PuMet 10	ND	46	–	–
Zhou [8]	25	37	Ab3	Semi-quantitative (M and/or Cy)	+	FISH	PT 44 PuMet 58	67	NS	NS	NA
Kilpatrick [17]	41	41	CB11 Oncor	Semi-quantitative (M and/or Cy)	+	–	0	ND	NA	NA	NA
Maitra [19]	21	21	AO485	Semi-quantitative (M)	+	FISH	0	0	NA	NA	NA
Thomas [20]	33	66	AO485	Semi-quantitative (M)	+	RT-PR	0	0	NA	NA	NA
Our study	33	27	Herceptest®	Herceptest® guidelines; (M)	+	Real-time PCR FISH	4[a]	0	NA	NA	NA

M, membrane staining; Cy, cytoplasmic 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; ND, no assay done; NS, relationship non-significant.

[a] Can be regarded as false-positive (see text).

Validation of the IHC HER2 results by the use of other assays was done in four out of the eight studies [5,8,19,20]. Validation assays included immunoblotting (IB), single-stranded conformation polymorphism (SSCP) and Southern blotting (SB)[5], RT-PCR [20] and FISH [8,19]. Except for one study that used FISH [8], 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 assesses 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 HER2 gene rearrangements in seven of 12 tested samples, but unusual criteria were used to define the *HER-2* gene amplification [8]. 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 *HER-2* gene amplification and aneusomy of chromosome 17 [41], which frequently occurs in OS [42]. Furthermore, *HER-2* gene amplification in HER2-overexpressing breast cancers is usually observed in most of the tumour cells [41].

Thus, to conclude, our results show that HER2 does not play a role in the tumour biology of OS and that pilot studies using trastuzumab, as a drug with potential tumour-inhibiting properties, are not likely to benefit patients with this bone tumour.

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, 264–270.
2. Davis AM, Bell RS, Goodwin PJ. Prognostic factors in osteosarcoma: a critical review. *J Clin Oncol* 1994, **12**, 423–431.
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* 2002, **20**, 776–790.
4. Hauben EI, Weeden S, Pringle J, Van Marck EA, Hogendoorn PCW. 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* 2002, **38**, 1218–1225.
5. Onda M, Matsuda S, Higaki S, *et al*. ErbB-2 expression is correlated with poor prognosis for patients with osteosarcoma. *Cancer* 1996, **77**, 71–78.
6. Gorlick R, Huvos AG, Heller G, *et al*. Expression of HER2/erbB-2 correlates with survival in osteosarcoma. *J Clin Oncol* 1999, **17**, 2781–2788.
7. Akatsuka T, Wada T, Kokai Y, *et al*. ErbB2 expression is correlated with increased survival of patients with osteosarcoma. *Cancer* 2002, **94**, 1397–1404.
8. Zhou H, Randall RL, Brothman AR, Maxwell T, Coffin CM, Goldsby RE. Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. *J Pediatr Hematol Oncol* 2003, **25**, 27–32.
9. 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* 1985, **230**, 1132–1139.
10. Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986, **232**, 1644–1646.
11. Hynes NE, Stern DF. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* 1994, **1198**, 165–184.
12. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* 1987, **237**, 178–182.
13. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987, **235**, 177–182.
14. Pierce JH, Arnstein P, DiMarco E, *et al*. Oncogenic potential of erbB-2 in human mammary epithelial cells. *Oncogene* 1991, **6**, 1189–1194.
15. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* 1985, **41**, 697–706.
16. 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* 1999, **18**, 2241–2251.
17. 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* 2001, **14**, 1277–1283.
18. Akatsuka T, Wada T, Kokai Y, Sawada N, Yamawaki S, Ishii S. Loss of ErbB2 expression in pulmonary metastatic lesions in osteosarcoma. *Oncology* 2001, **60**, 361–366.
19. Maitra A, Wanzer D, Weinberg AG, Ashfaq R. Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. *Cancer* 2001, **92**, 677–683.
20. Thomas DG, Giordano TJ, Sanders D, Biermann JS, Baker L. Absence of HER2/neu gene expression in osteosarcoma and skeletal Ewing's sarcoma. *Clin Cancer Res* 2002, **8**, 788–793.
21. Souhami RL, Craft AW, Eijken JWVD, *et al*. Randomised trial of two regimens of chemotherapy in operable osteosarcoma: a study of the European Osteosarcoma Intergroup. *Lancet* 1997, **350**, 911–917.
22. Bramwell V, Steward W, Nooy M, *et al*. Neoadjuvant chemotherapy with doxorubicin and cisplatin in malignant fibrous histiocytoma of bone: a European Osteosarcoma Intergroup Study. *J Clin Oncol* 1999, **17**, 3260–3269.
23. 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* 1994, **12**, 1842–1848.
24. Bramwell VH, Burgers M, Sneath R, *et al*. A comparison of two short intensive adjuvant chemotherapy regimens in operable osteosarcoma of limbs in children and young adults: the first study of the European Osteosarcoma Intergroup. *J Clin Oncol* 1992, **10**, 1579–1591.
25. Faneyte IF, Kristel PM, Van de Vijver MJ. Determining MDR1/P-glycoprotein expression in breast cancer. *Int J Cancer* 2001, **93**, 114–122.
26. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in

human mammary tumor cell lines by different molecular mechanisms. *EMBO J* 1987, **6**, 605–610.
27. Van de Vijver MJ, Devilee P, Cornelisse C, Peterse J, Nusse R. 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* 1987, **7**, 2019–2023.
28. King CR, Swain SM, Porter L, Steinberg SM, Lippman ME, Gelmann EP. Heterogeneous expression of erbB-2 messenger RNA in human breast cancer. *Cancer Res* 1989, **49**, 4185–4191.
29. Parkes HC, Lillycrop K, Howell A, Craig RK. C-erbB2 mRNA expression in human breast tumours: comparison with c-erbB2 DNA amplification and correlation with prognosis. *Br J Cancer* 1990, **61**, 39–45.
30. Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene* 1996, **13**, 63–72.
31. Farabegoli F, Ceccarelli C, Santini D, *et al*. c-erbB-2 over-expression in amplified and non-amplified breast carcinoma samples. *Int J Cancer* 1999, **84**, 273–277.
32. 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* 2001, **95**, 266–270.
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* 2001, **37**(Suppl. 1), 11–17.
34. Press MF, Hung G, Godolphin W, Slamon DJ. Sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. *Cancer Res* 1994, **54**, 2771–2777.
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* 2000, **18**, 3651–3664.
36. Kay EW, Walsh CJ, Cassidy M, Curran B, Leader M. C-erbB-2 immunostaining: problems with interpretation. *J Clin Pathol* 1994, **47**, 816–822.
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* 2001, **14**, 1079–1086.
38. Bilous M, Dowsett M, Hanna W, *et al*. Current Perspectives on HER2 Testing: A Review of National Testing Guidelines. *Mod Pathol* 2003, **16**, 173–182.
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* 1999, **11**, 311–317.
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* 1999, **45**, 1148–1156.
41. Kallioniemi OP, Kallioniemi A, Kurisu W, *et al*. ERBB2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 1992, **89**, 5321–5325.
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* 2003, **36**, 7–16.