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# Prognostic and therapeutic relevance of HER2 expression in osteosarcoma and Ewing's sarcoma

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**Abstract**

Expression of HER2 was evaluated by immunohistochemical techniques in 84 osteosarcoma (OS) and 113 Ewing's sarcoma (ES) paraffin-embedded tumour biopsies. *HER2* gene status was also assessed in a panel of cell lines as well as *in vitro* efficacy of trastuzumab (a humanised antibody directed against HER2) as single agent or in combination with the insulin-like growth factor I receptor (IGF-IR) IR3 antibody. Overexpression of HER2 was present in 32% of OS and 16% of ES and was significantly associated with the increased expression of P-glycoprotein, a surface molecule responsible for multidrug resistance. Event-free survival analyses revealed a prognostic value for HER2 and/or P-glycoprotein expression in OS, but not in ES. However, despite its prognostic relevance, no therapeutic effectiveness was observed pre-clinically for trastuzumab-driven therapy, in both OS or ES cell lines, unless the antibody was associated with anti-IGF-IR targeting strategies. Therefore, the therapeutic potential of trastuzumab in these neoplasms may be better exploited in combined treatments with anti-IGF-IR approaches.


*Keywords:* HER2; Osteosarcoma; Ewing's sarcoma; P-glycoprotein; Trastuzumab; IGF-IR

## 1. Introduction

The human epidermal growth factor receptor-2 (*HER2*) proto-oncogene (also known as *HER2 neu* or *c-erbB-2*) is located at 17q21 and encodes a 185-kDa transmembrane tyrosine kinase glycoprotein with extensive homology to the epidermal growth factor receptor [1–3]. Overexpression of *HER2* gene has been found to correlate with poor prognosis in a variety of human carcinomas, such as breast, ovarian, and lung cancers [4,5]. In some of these tumours, enhanced expression of HER2 has been associated with increased tumourigenicity and metastatic potential [6,7] or with resistance to chemotherapeutic agents [8,9]. In musculo-skeletal tumours, expression of HER2 at diagnosis has been analysed in soft-tissue sarcomas [10–12] and among bone neoplasms in Ewing's sarcoma (ES) [13,14] and osteosarcoma (OS) [15–21]. However, there is still a lack of general consensus regarding the possible prognostic impact of this marker in musculo-skeletal tumours, and it is still not clear whether HER2 may act jointly with other clinicopathological parameters to influence response to chemotherapy or clinical outcome.

In ES, HER2 has been evaluated by immunohistochemistry in a small series of clinical samples [13,14] or in few cell lines [22,23] and its biological relevance

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in this neoplasm remains to be clarified. More, but conflicting data, has been reported for OS. The presence of HER2 protein at diagnosis by immunohistochemical analysis has been found to correlate with worse clinical outcome [20] and is also associated with poor histological response to chemotherapy [15,16]. However, these findings have been recently questioned by other reports, which did not show any correlation between HER2 expression and poor treatment response. In some cases worse prognosis was not demonstrated [17,19] and one study even indicated a positive association between increased levels of HER2 and improved survival [18].

The purpose of this study was to investigate if the immunohistochemical detection of HER2 protein at diagnosis may identify high-risk groups of ES or OS patients by predicting clinical outcome. Moreover, on the basis of the suggested involvement of HER2 in resistance to chemotherapeutic agents, we also analysed the possible relationship between the expression of HER2 and the *MDR-1* gene product P-glycoprotein, which is a well established adverse prognostic marker in OS [24–27]. Finally, we used a panel of human ES and OS cell lines to further analyse the status of *HER2* gene in these tumour cells and to pre-clinically evaluate the effectiveness of HER2-tailored treatments, in order to determine whether this marker may be considered a possible new therapeutic target for these neoplasms.

## 2. Materials and methods

### 2.1. Patient eligibility and treatment protocols

The ES series included 283 patients who were referred to the Rizzoli Institute in Bologna (Italy) and entered into the REN-1, REN-2 or REN-3 neoadjuvant chemotherapy protocols. The inclusion criteria for these chemotherapy regimens were: primary tumour localised to the bone, younger than 40 years old, absence of metastases at diagnosis and no prior chemotherapy or surgical treatment for bone lesions. Patients were treated with pre- and post-operative chemotherapy according to the protocols mentioned above with minor modifications [28]. The local treatment consisted of surgery only, surgery followed by radiation therapy (combined treatment) or radiation therapy only. Histological response to chemotherapy was graded according to the classification of Picci and colleagues [29] as follows: grade I, macroscopic foci of viable tumour cells; grade II, isolated microscopic nodules of viable tumour cells and grade III, no presence of viable tumour cells. The extent of tumour necrosis was defined as total for grade III specimens and as non-total for grade I and II specimens.

The OS series included 154 patients who were referred to the Rizzoli Institute and entered in the IOR/OS-3a or IOR/OS-3b neoadjuvant chemotherapy protocols [30]. The inclusion criteria were: high-grade OS of the extremities, younger than 40 years of age, absence of metastases at the time of diagnosis, and no prior chemotherapy or surgical treatment for bone lesions. According to these protocols, patients were treated with pre-operative chemotherapy based on two cycles of high-dose methotrexate (10 g/m$^2$ in IOR/OS-3a and 12 g/m$^2$ in IOR/OS-3b) followed by a combination of cisplatin (120 mg/m$^2$) and doxorubicin (60 mg/m$^2$). On the basis of tumour necrosis evaluation after pre-operative chemotherapy, a good histological response was considered when the extent of tumour necrosis was 90% or greater. Post-operative chemotherapy used the same drugs as the pre-operative phase, with the addition of 10 g/m$^2$ ifosfamide for patients with poor histological response in IOR/OS-3a or for all patients in IOR/OS-3b protocol.

After surgery, both ES and OS patient specimens were evaluated in order to define the surgical margins and extent of viable tumour, which survived pre-operative chemotherapy. Radical or wide surgical margins were considered as adequate, whereas marginal or intralesional margins were classified as inadequate. Tumour volume was evaluated on CT-scans taken at the time of diagnosis as previously described [30]. According to volume, tumours were classified in two groups by using as cut-off the median value of 120 ml for ES or 150 ml indicated by the Cooperative Osteosarcoma Study Group (COSS) for OS [31]. After demission, patients were continuously followed and clinical data updated. Adverse events were defined as recurrence of the tumour at any site or death during remission. Event-free survival was calculated from the date of initial diagnosis.

### 2.2. Immunohistochemistry

All tumour tissue samples analysed by immunohistochemistry were obtained from biopsy specimens of untreated ES or OS patients. Immunohistochemical analysis for HER2 protein was performed on decalcified, paraffin-embedded samples of 113 ES and 84 OS. Among these, 87/113 ES and 80/84 OS were also analysed for P-glycoprotein presence. Expression of HER2 protein was assessed with two different monoclonal antibodies: 3B5 (1:900 dilution; BD Pharmingen, San Diego, CA) and CB11 (1:50 dilution; Novocastra, Newcastle, UK). In 43 cases of OS, expression of HER2 protein was also analysed with the Food and Drug Administration (FDA)-approved HercepTest™ kit (Dako Corp, Carpinteria, CA), according to the manufacturer's instructions. Expression of P-glycoprotein was assessed with three monoclonal antibodies, which react with different, mutually exclusive epitopes of this protein: JSB-1 (12.5 μg/ml; Sanbio, Uden, The Netherlands), MRK16

(0.003 μg/ml; Kamiya Biomedical, Thousand Oaks, CA) and C494 (0.2 μg/ml; Signet Laboratories, Dedham, MA). Immunohistochemistry for both HER2 and P-glycoprotein was performed on 4–6 μm paraffin-embedded tumour sections by using an avidin-biotin peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlinghame, CA, USA), as previously described [27]. For immunohistochemical detection of HER2 protein, sections were pre-treated with a citrate buffer solution (0.01 M citric acid and 0.01 M sodium citrate, pH 6.0) in a microwave oven at 750 W for three cycles of 5 min each. This treatment ensured antigen retrieval from the samples. For each specimen, both negative and positive controls for immunostaining were performed. In negative controls, the primary antibody was replaced with normal horse serum. Positive controls for HER2 included sections of one case of human breast cancer with amplified *HER2* and sections of normal kidney were used as positive control for P-glycoprotein expression. An additional positive control was also included for antigenicity of the tumour specimen by incubating it with the V9 anti-vimentin monoclonal antibody (Roche Molecular Biochemicals, Mannheim, Germany). Two independent blind investigators then scored the samples separately. For HER2, only specimens with more than 25% highly (+++) or moderately (++) positive cells were classified as positive. For P-glycoprotein, only samples with diffused immunostaining were classified as positive. Determinations of specimens that showed discrepancy between the different antibodies were repeated, and cases were classified on the basis of the most frequently recurring degree of immunostaining.

### 2.3. Cell lines

A panel of 12 human ES and 13 human OS cell lines were analysed. The ES cell lines SK-ES-1, RD-ES and SK-N-MC, the OS cell lines SaoS-2, U-2 OS and MG63, the human breast cancer cell line SK-BR-3 and the human ovarian cancer cell line SK-OV-3 were all obtained from the American Type Collection (Rockville, MD). The ES cell lines TC-71 and 6647 were kindly provided by T.J. Triche (Children's Hospital, Los Angeles, CA). All other ES sarcoma- (LAP-35, IOR/BRZ, IOR/CAR, IOR/NGR, IOR/BER, IOR/RCH, IOR/CLB) and OS cell lines (SARG, IOR/MOS, IOR/OS7, IOR/OS9, IOR/OS10, IOR/OS14, IOR/OS15, IOR/OS17, IOR/OS18, IOR/OS20) were established at the Laboratorio di Ricerca Oncologica, Istituti Ortopedici Rizzoli, (Bologna, Italy) and previously characterised [32,33]. All cell lines were routinely cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20 U/ml penicillin, 100 μg/ml streptomycin (SIGMA, St. Louis, MO), and 10% heat-inactivated fetal bovine serum (FBS) (Biowhittaker Europe, Verviers, Belgium) and maintained at 37 °C in a humidified 5% $CO_2$ atmosphere.

We also used transfected clones of the TC-71 ES cell line, which showed impaired insulin-like growth factor I receptor (IGF-IR) functions [34,35]. These cells were cultured in IMDM plus 10% FBS plus 500 μg/ml G418 (SIGMA Chemical Co, St. Louis, MO) to maintain stable transfectants. For TC-71 clones transfected with IGF-IR antisense expressing plasmid, cells were maintained at 39.6 °C for 72 h, a temperature reported to induce the expression of sense and antisense transcripts.

### 2.4. Fluorescence in situ hybridisation

Fluorescence *in situ* hybridisation (FISH) was performed on interphase nuclei of human ES and OS cell lines with the FDA-approved Pathvysion HER2/*neu* DNA probe (Abbott-Vysis, Downers Grove, IL), by following the manufacturer's guidelines. For the hybridisation step, a mixture of the two directly labeled probes LSI HER2/*neu* SpectrumOrange (specific for the *HER2* gene) and the CEP17 SpectrumGreen (specific for the alpha-satellite, centromeric DNA of chromosome 17) was applied to interphase nuclei specimens and incubated overnight at 37 °C. After post-hybridisation washes, nuclei were counterstained with DAPI. For each specimen, at least 100 nuclei were counted for both the *HER2* gene and CEP17 hybridisation signals. The results were reported as a ratio of the average *HER2* gene copy number to that of CEP17. Amplification of *HER2* gene was assigned only to specimens with a HER2/CEP17 signal ratio $\geqslant 2$.

### 2.5. Cytofluorometric analysis of HER2- and IGF-IR expression

HER2- and IGF-IR expression was evaluated by indirect immunofluorescence and flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA) by using the anti-HER2 monoclonal antibody TAB250 (Zymed Laboratories Inc., San Francisco, CA; dilution 1:400) or the anti-IGF-IR monoclonal antibody αIR3 (Oncogene Research Products, San Diego, CA; dilution 1:10).

### 2.6. In vitro efficacy of trastuzumab alone or in association with anti-IGF-IR treatments

To evaluate the effectiveness of trastuzumab (Herceptin, Roche Diagnostics GmbH, Mannheim, Germany), 20,000/$cm^2$ cells were seeded in IMDM 10% FBS. After 24 h, medium was changed with IMDM 1% FBS without (control) or with increasing doses of the drug (0.3–100 μg/ml). For combined *in vitro* treatments, 20,000 cells/$cm^2$ were seeded in 6-well plates in IMDM plus 10% FBS. After 24 h, medium was changed with

IMDM plus 1% FBS and cells were treated with trastuzumab (0.3–100 μg/ml) jointly with/out the neutralizing anti-IGF-IR αIR3 monoclonal antibody (1 μg/ml for OS cells and 30–100 ng/ml for ES cell lines). After 72 h (for ES cell lines) or 96 h (for OS cell lines) cell growth was evaluated on harvested cultures by trypan blue vital cell count.

### 2.7. Soft-agar assay

Anchorage-independent growth was determined in 0.33% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) with a 0.5% agarose underlay. Cell suspensions were plated in a semi-solid medium (IMDM plus 10% FBS containing 0.33% agarose) with or without trastuzumab (10–100 μg/ml; cells/dish 60 mm∅: 3300 and 10,000 for SK-N-MC and U-2 OS; 10,000 and 33,000 for SK-OV-3 and SK-BR-3). Dishes were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$ and colonies were counted after 14 days.

### 2.8. Statistical analysis

Two-tailed Fisher's exact test was used to evaluate the statistical association between two variables. Differences among means were analysed using two-sided Student's *t* test. The effects of drug combination were analysed by using the fractional product method. Kaplan-Meier and log-rank methods were used to draw and evaluate the significance of survival curves, respectively.

## 3. Results

### 3.1. Clinical series

The two groups of 113 ES and 84 OS patients included in this study are representative of the whole series of 283 ES and 154 OS patients who underwent the chemotherapy protocols considered here. In fact, no significant differences were found between the clinicopathological characteristics of the whole group of patients included in these treatment protocols and the subgroups considered for immunohistochemical analyses (Tables included as supplementary materials).

### 3.2. Analysis of HER2 and P-glycoprotein expression in association with clinicopathological features

Consistent with previous observations [17,20,36], HER2-positive cases exhibited focal to diffuse cytoplasmic immunostaining (Fig. 1), without concomitant membranous staining. In ES, presence of HER2 was revealed in 18/113 cases (16%). Immunohistochemistry for HER2 showed a complete concordance between the 3B5 and CB11 monoclonal antibodies ($P < 0.0001$; two-tailed Fisher's exact test). Eighty-seven of these 113 cases were also immunostained for P-glycoprotein. A positive immunoreaction for P-glycoprotein was found in 27/87 samples (31%). A significant association was found between HER2 and P-glycoprotein expression ($P < 0.0001$; two-tailed Fisher's exact test), with a concordance between these two markers in 68/87 cases (78%). Among the 19 discordant cases, 17 were positive for P-glycoprotein and negative for HER2, whereas two were positive for HER2 and negative for P-glycoprotein.

In OS, presence of HER2 was revealed in 27/84 cases (32%), with a concordance between the 3B5 and CB11 monoclonal antibodies in 78% of cases ($P < 0.0001$; two-tailed Fisher's exact test). In 43 cases, expression of HER2 was also analysed by the DAKO HercepTest, a semi-quantitative immunohistochemical assay to detect increased levels of HER2 protein in paraffin-embedded specimens. A positive result from HercepTest was found in 12/43 cases (28%), a frequency similar to that obtained with the 3B5 and CB11 monoclonal antibodies. Concordance between 3B5/CB11 and HercepTest immunohistochemical results was found in 29/43 cases (67%; $P < 0.01$; two-tailed Fisher's exact test). Among the 14 discordant cases, 12 resulted positive for 3B5/CB11 and negative for HercepTest, whereas only two were negative for 3B5/CB11 and positive for HercepTest.

For 80 of the 84 OS analysed for HER2, immunohistochemical results for P-glycoprotein were also obtained. Expression of P-glycoprotein was found in 39/80 cases (49%). In agreement with the observations in ES, in OS also, expressions of HER2 and P-glycoprotein were found to be significantly associated ($P < 0.0001$; two-tailed Fisher's exact test). Concordance between these two markers was found in 58/80 cases (73%; $P < 0.0001$; two-tailed Fisher's exact test). Among the 22 discordant cases, 17 were positive for P-glycoprotein and negative for HER2, whereas five were negative for P-glycoprotein and positive for HER2.

Both in ES and in OS, HER2 or P-glycoprotein expression did not significantly correlate with other clinicopathologic features (gender, age, histological subtype, site, chemotherapeutic protocol, local therapy, surgery, surgical margins, tumour volume at diagnosis, or tumour necrosis after pre-operative chemotherapy; data not shown), indicating that these two markers are independent from the clinicopathological characteristics of the tumours.

### 3.3. Clinical outcome and survival analysis

The prognostic value of HER2, P-glycoprotein and clinicopathological variables was analysed in relation to relapse rate and clinical outcome. For all patients, event-free survival was calculated from the date of initial

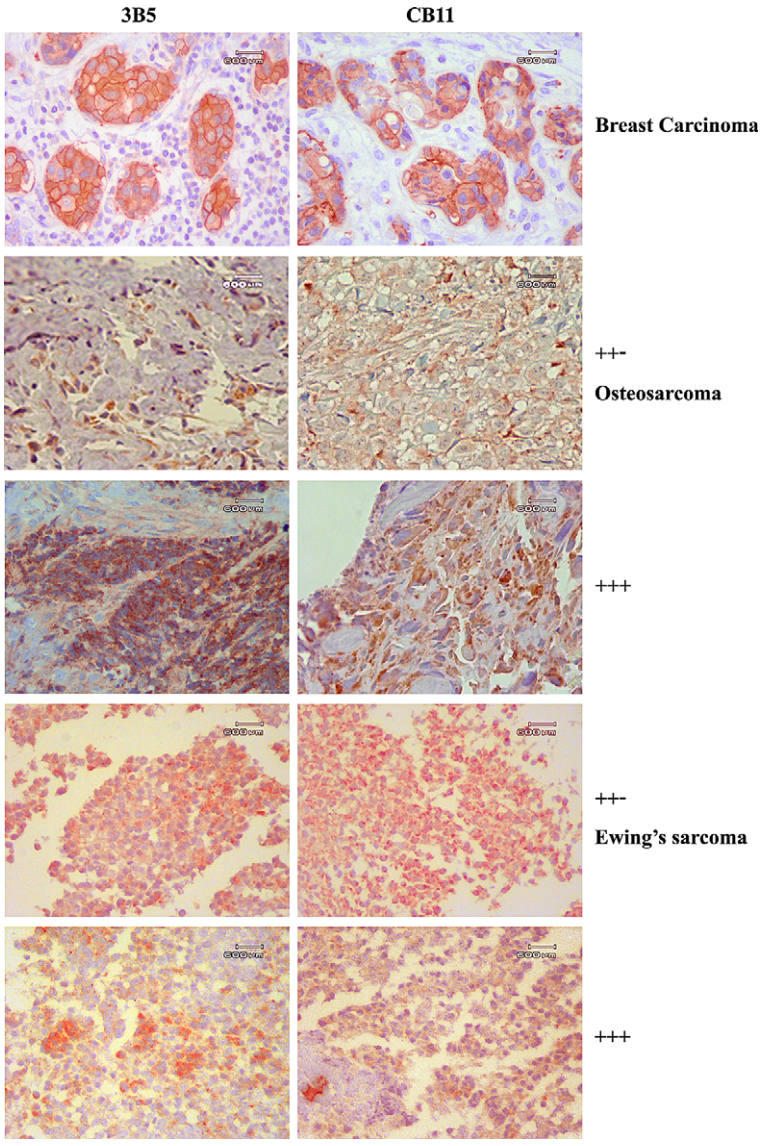


Fig. 1. Immunostaining for HER2 protein in Ewing's sarcoma and osteosarcoma tissue samples after immunohistochemical analysis with the 3B5 or CB11 monoclonal antibodies. Breast carcinoma tissue samples were used as positive controls. The degree of immunostaining was scored as highly (+++) or moderately positive (++) on the basis of levels of antibody binding. Original magnification, 250×.

diagnosis. Adverse events included relapse of the tumour at any site or death during remission.

The median follow-up of the 113 ES patients was 7.0 years (range 3.6–16.3 years), and adverse events occurred in 60/113 patients (53%). As shown in Table 1, univariate analysis demonstrated that a higher relapse rate was significantly associated with age older than 12 years ($P < 0.023$ two-tailed Fisher's exact test), inadequate surgical margins ($P < 0.047$ two-tailed Fisher's exact test), and non-total tumour necrosis ($P < 0.049$ two-tailed Fisher's exact test). Event-free survival analyses further confirmed the association of age older than 12 years, inadequate surgical margins, and non-total tumour necrosis with a worse clinical outcome ($P < 0.05$ by log-rank test for all these three parameters; data not shown).

The analysis of the prognostic relevance of HER2 alone or in association with P-glycoprotein in these series of ES patients did not reveal any significant association with relapse rate (Table 1). Event-free survival analyses did not show any significant difference between the groups of HER2-positive and HER2-negative patients (Fig. 2(a)), further confirming the lack of association between these two markers and clinical outcome. Similar results were obtained also in relation to the expression of P-glycoprotein (Fig. 2(b)) or to both HER2 and P-glycoprotein (Fig. 2(c)). Therefore, neither HER2- nor P-glycoprotein expression appear to have a prognostic value in ES.

Among the 84 OS analysed for HER2, complete outcome information was available for 83 patients, whose median follow-up was of 8.7 years (range 6.6–10.9

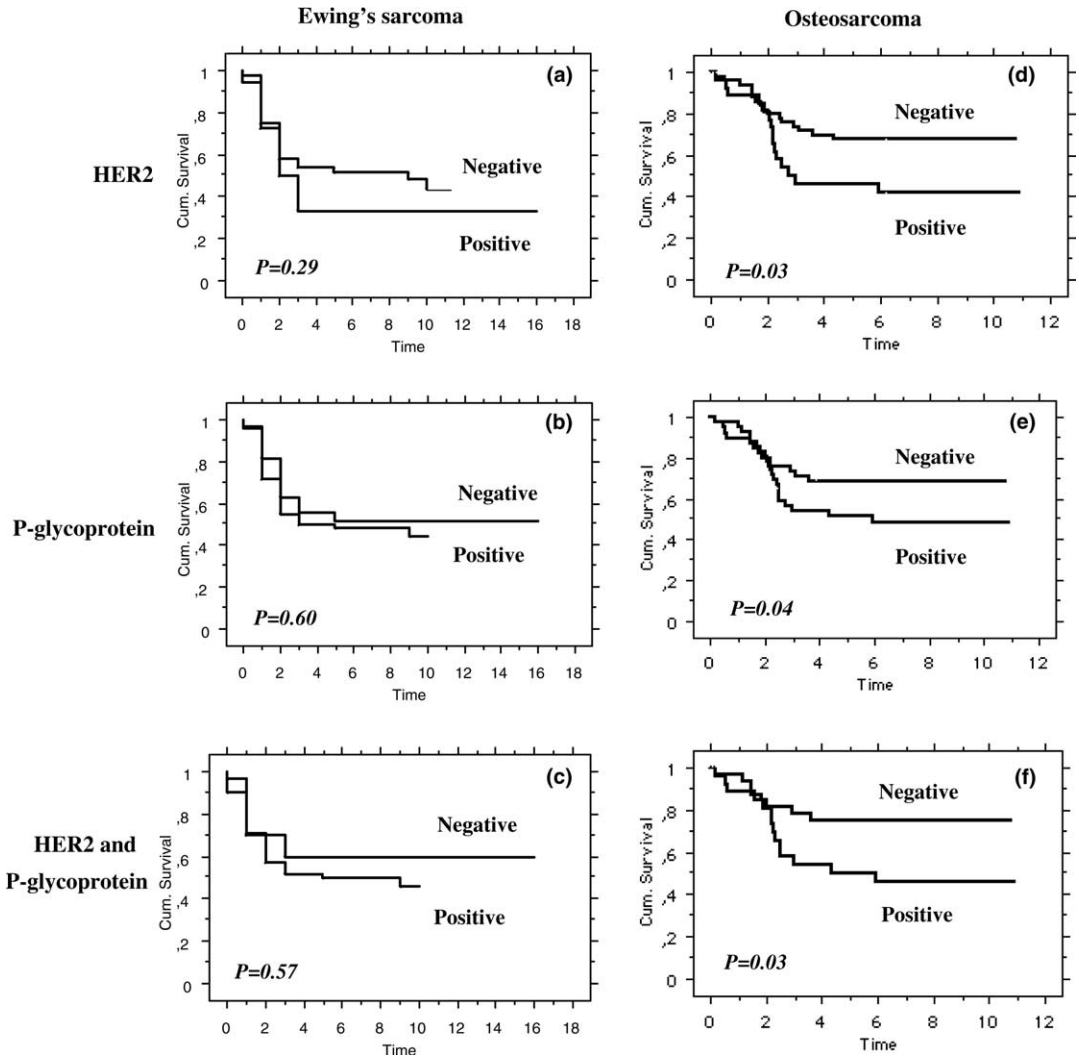


Fig. 2. Event-free survival curves in 113 Ewing's sarcoma and 83 osteosarcoma patients according to the expression of HER2 (a and d) or P-glycoprotein (b and e), as well as in relation to the simultaneous expression of both markers (c and f). Survival curves were drawn and evaluated by using the Kaplan-Meier and log-rank methods.

years). As shown in Table 2, adverse events occurred in 31/83 patients (37%). A higher relapse rate resulted to be significantly associated with both HER2 expression ($P = 0.01$; two-tailed Fisher's test), and P-glycoprotein ($P = 0.04$; two-tailed Fisher's test), or to both HER2 and P-glycoprotein ($P = 0.04$; two-tailed Fisher's test). Accordingly, event-free survival analyses showed that only HER2 and P-glycoprotein were significantly associated with a worse clinical outcome, either when considered alone or together (Fig. 2(d)–(f)). In this study, differently from ES, both HER2 and P-glycoprotein emerged to have a remarkable prognostic relevance in OS.

### 3.4. HER2 status in ES and OS cell lines

HER2 overexpression is often due to gene amplification in breast cancer. However, in other tumours the frequency of HER2 overexpression is often greater than predicted by gene amplification data, suggesting that overexpression in these neoplasms is due to gene deregulation rather than amplification.

HER2 status was assessed in a panel of 12 ES and 13 OS cell lines. By flow cytometry, expression of HER2 was found in 10 out of 12 (83%) ES cell lines and in eight out of 13 (62%) OS cell lines (Fig. 3). Protein expression in both ES and OS cell lines appears to be higher than in clinical samples, but these could reflect the higher sensitivity of flow cytometry compared to immunohistochemistry. However, in all these cell lines, the intensity of fluorescence was generally lower than that observed in the breast cancer cell line SK-BR-3 and in the ovarian cancer cell line SK-OV-3, which were used as positive controls.

In agreement with these observations, none of the ES or OS cell lines showed amplification of *HER2* gene by FISH analyses (Table 3). The mean *HER2* gene/CEP17 copy number ratio ranged between 0.5 and 1.0 in ES and

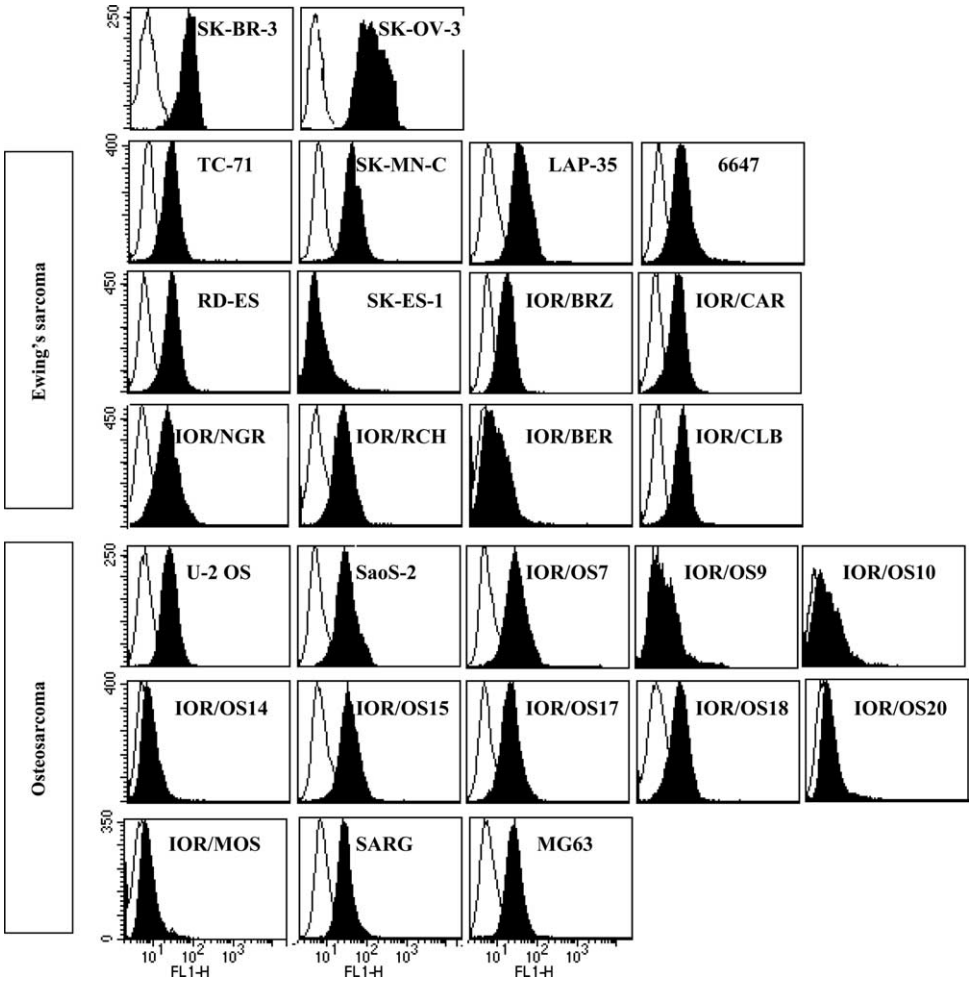


Fig. 3. Cytofluorometric analysis of HER2 expression in human Ewing's sarcoma and osteosarcoma cell lines. Open profile: cells stained with secondary antibody only. Solid profile: cells stained with the anti-HER2 antibody. Data from an experiment representative of at least two similar determinations are shown.

between 0.4 and 1.0 in OS cell lines, respectively. Only the positive controls SK-BR-3 and SK-OV-3 cell lines showed amplification of *HER2* gene. Fig. 4 shows the *HER2* gene amplification in SK-BR-3 and SK-OV-3 cells, together with two representative ES and OS cell lines (LAP-35 and SARG, respectively) without evidence of *HER2* gene amplification. A small series of 10 clinical samples were also analysed for *HER2* gene status by FISH, but no increase of *HER2* copy number was observed, further confirming the flow cytometry results (data not shown).

### 3.5. In vitro effects of trastuzumab in ES and OS cells

Independently from the molecular mechanisms responsible for HER2 overexpression, the findings obtained in this study indicated that approximately 15–20% of ES patients and 30% of OS patients were positive for HER2 in their primary tumours. Therefore, it was possible that they could be considered for treatment with trastuzumab, a humanised antibody directed against the extracellular domain of the tyrosine kinase receptor HER2, that is known for its clinical activity against HER2 overexpressing tumours [37,38]. Fig. 5(a) shows the inhibitory growth effects of trastuzumab against three representative ES or OS cell lines. Apart from the SaoS-2 OS cell line, which showed percentages of growth inhibition similar to those obtained with the positive control SK-BR-3 breast carcinoma cells, in all the other cell lines a maximum of 20% growth inhibition was found, which is a modest reduction in monolayer conditions. In addition, when we analysed the effects of trastuzumab in soft-agar (Fig. 5(b)), we found that the drug was effective in SK-BR-3 and SK-OV-3 cell lines, whereas no effect at all was observed in ES and OS cells, further supporting its therapeutic role in breast and ovarian cancers but not in bone tumours.

Since IGF-IR has recently been shown to be involved in the resistance to trastuzumab [39,40] and this receptor is known to play a major role in the pathogenesis and progression of ES and OS [32,34,35,41], we investigated whether strategies targeting IGF-IR would potentiate

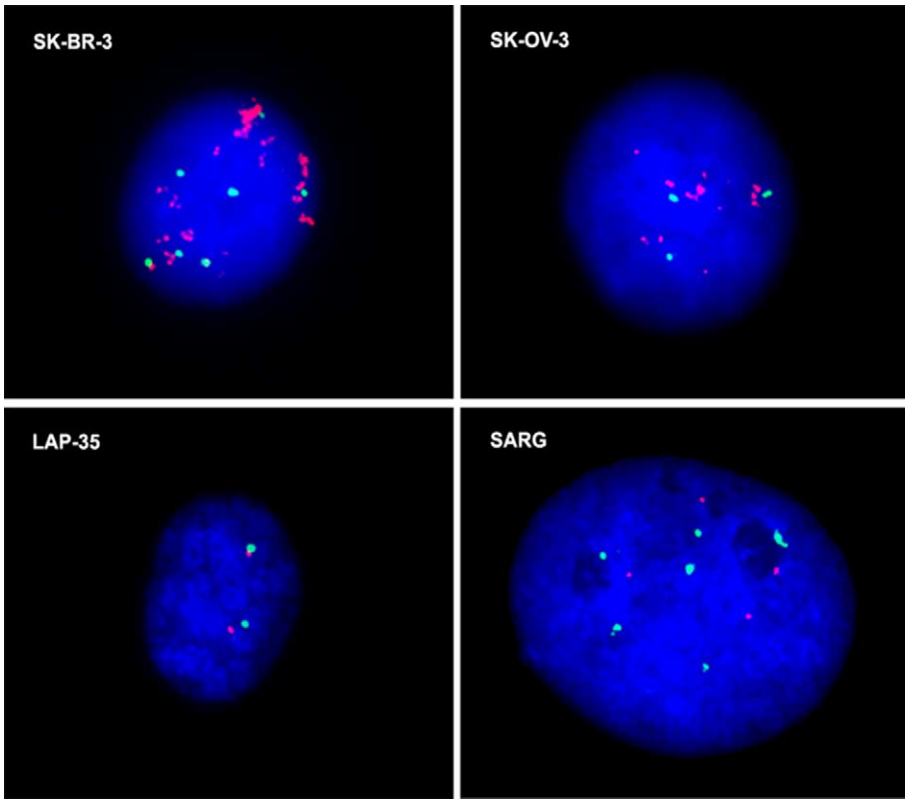


Fig. 4. Detection of *HER2* gene copy number (*red signals*) in relation to chromosome 17 centromeric signals (*green signals*) by fluorescence *in situ* hybridisation (FISH). Amplification of *HER2* gene is present in positive controls SK-OV-3 (ovarian carcinoma) and SK-BR-3 (breast carcinoma) nuclei. Original magnification, 600×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the growth inhibitory action of trastuzumab. Combined experiments with the neutralizing anti-IGF-IR antibody αIR3 and trastuzumab were performed on two ES and two OS cells lines that express both HER2 (Fig. 3) and IGF-IR (Fig. 6). The SK-BR-3 breast cancer cell line, which expresses HER2 but barely IGF-IR (Figs. 3 and 6), was used as negative control. Indeed, the modest trastuzumab-induced growth inhibition observed in ES and OS cells was enhanced by αIR3 (Fig. 7), and an additive growth suppression effect due to the combination treatment was observed. The role of IGF-IR targeting strategies to increase the growth-inhibitory activity of trastuzumab was also confirmed by using TC-71 derived clones, in which IGF-IR functions are impaired by using antisense or dominant negative IGF-IR mutants [34,35]. Clones expressing antisense sequences or producing an IGF-IR protein mutated at the ATP-binding site in the tyrosine kinase domain were more sensitive to trastuzumab compared to control cells (data not shown).

## 4. Discussion

Overexpression of HER2, is often associated with gene amplification and has been reported for several human tumours including breast, ovary, stomach, salivary gland, colon, kidney and bladder cancers [4,5]. Although HER2 expression appears to be a relatively frequent event in epithelial tumours, it has also been demonstrated in neoplasms of mesenchymal origin, including bone and soft-tissue sarcomas. In bone tumours, several studies on HER2 have been performed for OS and ES, the two most common neoplasms of the skeleton, but the findings reported so far are rather contradictory [13–21]. The reported percentages of protein expression ranged widely and no general consensus has been achieved on the prognostic value of HER2 in these two neoplasms, indicating that HER2 altered expression and role in sarcoma progression is not clear and requires additional studies.

In particular for OS, the data reported so far concerning HER2 are rather controversial, ranging from studies in which HER2 increased expression was detected in 10–63% of patients [15,16,18] to others in which it was very uncommon or even absent [17,19,21]. Moreover, as extensively discussed by Anninga and colleagues [21], inconsistent findings have also been reported concerning HER2 status and its prognostic significance. In fact in some studies, the increased levels of HER2 protein at diagnosis resulted in an association with poor histological response to chemotherapy and/or

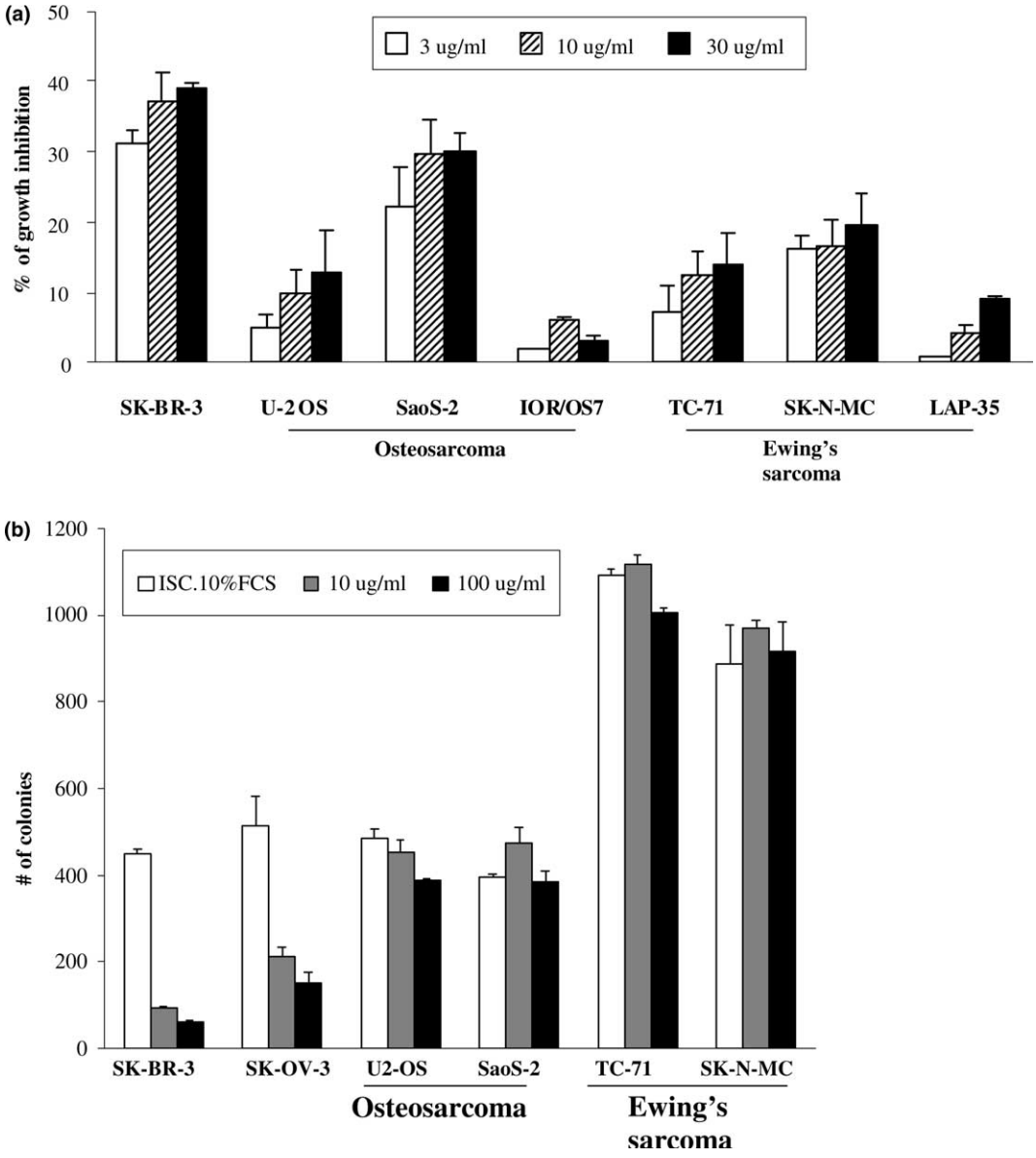


Fig. 5. *In vitro* sensitivity of osteosarcoma and Ewing's sarcoma cell lines to trastuzumab: (a) Monolayer assay. Cells were exposed for 72 h to different doses of the drug. (b) Soft-agar assay. Cell suspensions were plated in a semi-solid medium (IMDM plus 10% FBS containing 0.33% agarose), with or without trastuzumab, and colonies were counted after 14 days. All results show the mean of three independent experiments ± standard error.

a worse clinical outcome [15,16,20], whereas other reports either did not show any correlation between HER2 and poor treatment response or prognosis [17,19] or indicated a positive prognostic value for increased HER2 expression at diagnosis [18].

We can only speculate about the possible reasons for this lack of concordance. Discrepancies may have arisen from several sources such as differences in technical approach, tissue processing, anti-HER2 antibodies used for the assays, result interpretation, sample series size and treatment protocols. All these parameters vary from study to study and the impact of each of them may have significantly influenced the conclusions of each report.

In this study, we assessed the expression of HER2 in 113 ES and 84 OS homogeneously treated patients, which represent a rather large series for these rare tumours, to define the prognostic relevance of this marker. Moreover, since some studies reported that HER2-positive cells may represent biologically aggressive or drug resistant cell populations [6–9,20], the expression of HER2 was also analysed in relation to that of P-glycoprotein, which is responsible for multidrug resistance and is a well known adverse prognostic marker for OS [24–27].

The immunohistochemical detection of HER2 was performed with the extensively validated 3B5 and

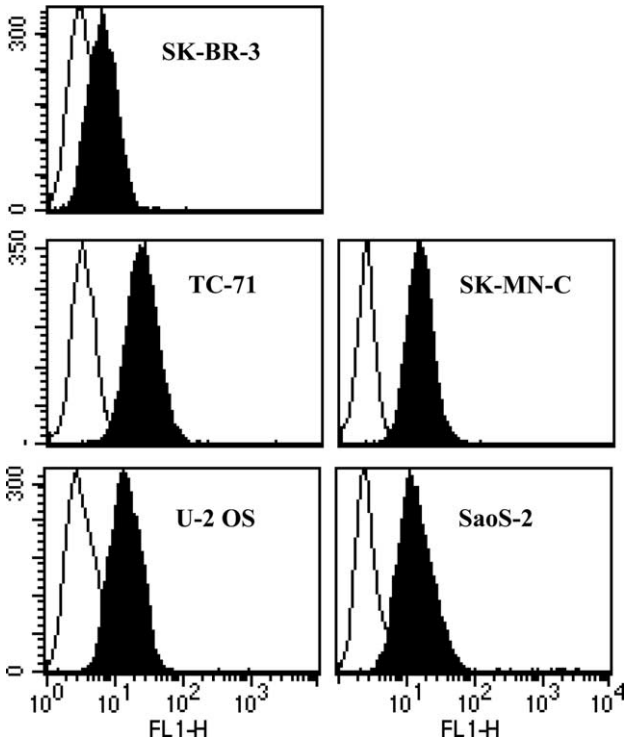


Fig. 6. Flow cytometry analysis of IGF-IR expression in human Ewing's sarcoma (TC-71 and SK-N-MC) and osteosarcoma (U-2 OS and SaoS-2) cell lines. Open profile: cells stained with secondary antibody only. Solid profile: cells stained with the anti-IGF-IR antibody. Data from an experiment representative of at least two similar determinations are shown.

CB11 monoclonal antibodies [42], for which no cross-reactivity with P-glycoprotein or other cellular antigens has been reported [43]. Differently from what has been observed in carcinomas, both in ES and OS, the HER2 was present in the cytoplasm of positive cases. In few cases, it was associated with a concomitant membranous immunostaining. These findings are consistent with previous observations [17,20,36], despite the fact that that reliability of HER2-cytoplasmic positivity in OS has been recently questioned [21]. This evidence may be explained by taking into account that, when HER2 protein is detected by immunohistochemistry, its pattern of staining is faint and diffuse and appears to be associated with a cytoplasmic rather than with a cell surface reaction, as also demonstrated by Hughes and colleagues [36] in both paraffin-embedded OS cell lines and clinical samples. This feature may be due to the much lower levels of HER2 protein that are reached in bone tumour cells compared to breast or ovarian cancers, as also indicated by the flow cytometric data of this study and by Hughes and colleagues [36], which may prevent a clear immunodetection of the protein on the cell surface. Alternatively, it may derive from histological differences between bone tumour- and carcinoma cells, which may allow a better staining of the cell membrane in the latter case.

In this study, increased levels of HER2 protein were found in 16% of ES and 32% of OS, with a significant concordance between the 3B5 and CB11

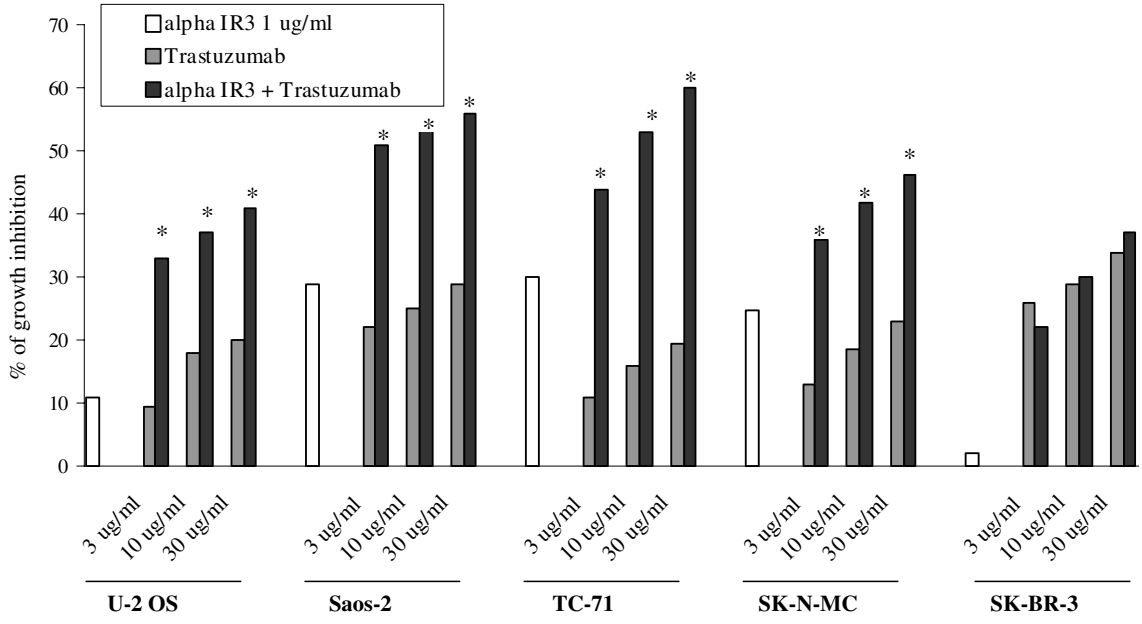


Fig. 7. Inhibitory growth effects of trastuzumab treatment in combination with the neutralising anti-IGF-IR antibody αIR3 in osteosarcoma and Ewing's sarcoma cell lines. Cells were treated with trastuzumab at the indicated concentrations, alone or in association with αIR3 for a total of 72 h, starting from the first day after seeding. *, $P < 0.05$ (Student's $t$ test) compared to the corresponding dose of each single drug.

immunohistochemical results ($P < 0.0001$), therefore indicating that inherent overexpression of this receptor can be present in these neoplasms. Fourty-three OS were also immunostained with the HercepTest kit, an additional and widely used method for HER2 protein immunodetection. HercepTest revealed a positive immunostaining for HER2 protein in 28% of cases, showing a concordance with the immunohistochemical results obtained by 3B5 and CB11 monoclonal antibodies in 67% of cases ($P < 0.05$). It is interesting to note that among the 14 discordant cases, 12 were positive for 3B5 and CB11 but negative for HercepTest, suggesting that the latter method may identify only cases with higher levels of HER2 protein, as also indicated by others [16].

Immunohistochemical analyses also revealed a positive correlation between HER2 and P-glycoprotein overexpression in both ES and OS ($P < 0.0001$), which is a new finding of this study that should be validated in future studies by using additional methods. The relationship between P-glycoprotein and HER2 overexpression is not due to technical artefacts, such as non-specificity of the antibodies employed in this study, because no cross-reaction has been demonstrated between them [43]. Moreover, the fact that, both in ES and OS, discordant cases resulted to be mostly positive to P-glycoprotein and negative to HER2 suggests that overexpression of P-glycoprotein may appear earlier than that of HER2, which may be more closely related to increased cellular aggressiveness or tumour progression.

With respect to the prognostic impact of HER2 and P-glycoprotein overexpression, our findings differ between ES and OS. In ES, analysis of clinical outcome revealed an adverse prognostic value for age older than 12 years, inadequate surgical margins, and non-total tumour necrosis, all of which are associated with both a higher relapse rate and a worse clinical outcome, in agreement with previous reports [28]. On the other hand, neither HER2 nor P-glycoprotein expression, alone or in association, showed no prognostic value and appeared not to be biologically relevant for the outcome of this tumour, substantially confirming previously reported sporadic data, which have been separately obtained for HER2 [13,14] and/or P-glycoprotein [44–46] expression on small series of ES.

Differently from ES, our data demonstrate that both HER2 and P-glycoprotein have prognostic value in OS. In fact, event-free survival analyses showed that, among all the clinicopathological parameters considered here, only overexpression at diagnosis of HER2 or P-glycoprotein, as well as of both molecules, was significantly associated with a higher relapse rate and a worse clinical outcome. We further demonstrated the value of these markers in predicting OS prognosis and response to therapy and revealed that HER2 and P-glycoprotein may also act together as adverse prognostic factors in determining a worse prognosis in patients who present inherent increased expression of both these molecules at diagnosis. In our opinion, simultaneous overexpression of HER2 and P-glycoprotein in OS may identify a subset of tumours characterised by a peculiar pattern of gene expression related to poor response to drugs or a more aggressive biological behaviour. A molecular portrait of this subset of tumours is under definition.

Differential levels of HER2 expression together with the involvement of HER2 in tumour progression indicate this receptor as a possible target for therapeutic approaches. Indeed, both ES and OS are relatively rare sarcomas with limited therapeutic options, in particular for relapsed patients, who are extremely difficult to treat because of their unresponsiveness to conventional chemotherapy. The availability of trastuzumab (also known as Herceptin), the humanised antibody specific to HER2 protein that is currently used in the treatment of advanced breast cancer [47], may represent an opportunity for the 20–30% of ES and OS patients over-expressing HER2 receptor at the time of diagnosis, provided that its effectiveness in inhibiting HER2 overexpressing sarcoma cells is proved. Therefore, we analysed the *in vitro* therapeutic impact of trastuzumab on a panel of human ES and OS cell lines. Although the expression of HER2 receptor was found in the majority of cell lines considered here, the growth-inhibitory effects of trastuzumab was definitely low in monolayer- and absent in soft-agar culture conditions. These rather modest results obtained in our experimental models indicate that HER2 is not a good therapeutic target for these two bone tumours.

Evaluation of *HER2* gene copy number status in sarcoma cell lines by FISH clearly indicated that none of the ES and OS cell lines considered here showed amplification of *HER2* gene. These findings, in agreement with previous reports [15,19,23], indicated that HER2 overexpression in both ES and OS cells was independent from gene amplification. The lack of gene amplification may be, at least partly, responsible for the scarce anti-tumour effects of trastuzumab in these neoplasms, since data derived from breast cancer indicate that efficacy of trastuzumab treatment is strictly related to the increased HER2 protein level associated with amplification of *HER2* gene [48].

A second reason for ES and OS cells being substantially resistant to trastuzumab may be related to the increased level of IGF-IR signaling in these cells. Both ES and OS are indeed characterised by the presence of an IGF-IR-mediated autocrine loop that greatly influences growth, migratory and metastatic ability of these cells [32,34,35,41]. Recent reports have clearly

demonstrated that IGF-IR signaling adversely interferes with the action of trastuzumab on cell growth [39,40]. Accordingly, treatments with the neutralizing anti-IGF-IR monoclonal antibody αIR3 of ES and OS cells, as well as of cells genetically modified to down-modulate IGF-IR expression, showed significantly higher sensitivity to trastuzumab. Although other mechanisms may be responsible for trastuzumab resistance, we further confirmed the critical role of IGF-IR in regulating the growth-inhibitory action of trastuzumab.

In conclusion, with this paper, we have contributed to clarify the role and importance of HER2 in bone tumours. In particular, we have identified an adverse role of HER2 expression in the progression of OS but not in ES. Interestingly, in OS, HER2 overexpression appears to be associated with the increased expression of P-glycoprotein, a surface molecule responsible for multidrug resistance that is also associated with a worse prognosis. The hypothesis that HER2 and P-glycoprotein overexpression could be peculiar for a subset of OS, which could be also characterized by the expression of other genes that affect patients' outcome is under investigation. Disappointingly, despite the prognostic value of HER2 overexpression, OS patients inherently expressing this molecule appear to have low chances to benefit from trastuzumab-driven therapy. Our pre-clinical studies have clearly indicated that this drug shows very limited growth-inhibitory actions against both OS and ES cells. In addition, we have identified the lack of gene amplification and the increased levels of IGF-IR signaling as two possible mechanisms of resistance to trastuzumab.

## Ethical permission statement

Patients were required to provide written informed consent to participate in the study.

## Conflict of interest statement

The Authors indicated no potential conflicts of interest and disclose any financial and personal relationships with other people or organisations that could inappropriately have influenced or biased this work.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2005.03.015.

## References

1. 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.
2. King CR, Kraus MH, Aaronson SA. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* 1985, **229**, 974–976.
3. 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* 1986, **232**, 1644–1656.
4. Slamon DJ, Godolphin W, Jones LA, *et al*. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989, **244**, 707–712.
5. Hynes NE, Stern DF. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* 1994, **1198**, 165–184.
6. Yu D, Wolf JK, Scanlon M, *et al*. Enhanced c-erbB-2/neu expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res* 1993, **53**, 891–898.
7. Yu D, Wang SS, Dulski KM, *et al*. c-erbB-2/neu overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. *Cancer Res* 1994, **54**, 3260–3266.
8. Tsai CM, Yu D, Chang KT, *et al*. Enhanced chemoresistance by elevation of p185neu levels in HER-2/neu-transfected human lung cancer cells. *J Natl Cancer Inst* 1995, **87**, 682–684.
9. Yu D, Liu B, Tan M, *et al*. Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. *Oncogene* 1996, **13**, 1359–1365.
10. Merimsky O, Issakov J, Schwartz I, *et al*. Lack of ErbB-2 oncogene product overexpression in soft tissue sarcomas. *Acta Oncol* 2002, **41**, 366–368.
11. Foster H, Knox S, Ganti AK, *et al*. HER-2/neu overexpression detected by immunohistochemistry in soft tissue sarcomas. *Am J Clin Oncol* 2003, **26**, 188–191.
12. Sato T, Peiper M, Heinecke A, *et al*. Expression of HER2/neu does not correlate with survival in soft tissue sarcoma. *Onkologie* 2003, **26**, 268–271.
13. Thomas DG, Giordano TJ, Sanders D, *et al*. Absence of HER2/neu gene expression in osteosarcoma and skeletal Ewing's sarcoma. *Clin Cancer Res* 2002, **8**, 788–893.
14. George E, Niehans GA, Swanson PE, *et al*. Overexpression of the c-erbB-2 oncogene in sarcomas and small round-cell tumours of childhood. An immunohistochemical investigation. *Arch Pathol Lab Med* 1992, **116**, 1033–1035.
15. 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.
16. 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.
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, *et al*. ErbB2 expression is correlated with increased survival of patients with osteosarcoma. *Cancer* 2002, **94**, 1397–1404.
19. Maitra A, Wanzer D, Weinberg AG, *et al*. Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. *Cancer* 2001, **92**, 677–683.
20. 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* 2003, **25**, 27–32.
21. Anninga JK, van de Vijver MJ, Cleton-Jansen AM, *et al*. Overexpression of the HER-2 oncogene does not play a role in high-grade osteosarcomas. *Eur J Cancer* 2004, **40**, 963–970.
22. Zhou Z, Jia SF, Hung MC, *et al*. E1A sensitizes HER2/neu-overexpressing Ewing's sarcoma cells to topoisomerase II-targeting anticancer drugs. *Cancer Res* 2001, **61**, 3394–3398.
23. Ye D, Maitra A, Timmons CF, *et al*. The epidermal growth factor receptor HER2 is not a major therapeutic target in Ewing sarcoma. *J Pediatr Hematol Oncol* 2003, **25**, 459–466.
24. Baldini N, Scotlandi K, Barbanti-Bròdano G, *et al*. Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome. *New Eng J Med* 1995, **333**, 1380–1385.
25. Chan HS, Grogan TM, Haddad G, *et al*. P-glycoprotein expression: critical determinant in the response to osteosarcoma chemotherapy. *J Natl Cancer Inst* 1997, **89**, 1706–1715.
26. Pakos EE, Ioannidis JP. The association of P-glycoprotein with response to chemotherapy and clinical outcome in patients with osteosarcoma. A meta-analysis. *Cancer* 2003, **98**, 581–589.
27. Serra M, Scotlandi K, Reverter-Branchat G, *et al*. Value of P-glycoprotein and clinicopathologic factors as the basis for new treatment strategies in high-grade osteosarcoma of the extremities. *J Clin Oncol* 2003, **21**, 536–542.
28. Bacci G, Ferrari S, Bertoni F, *et al*. Prognostic factors in nonmetastatic Ewing's sarcoma of bone treated with adjuvant chemotherapy: analysis of 359 patients at the Istituto Ortopedico Rizzoli. *J Clin Oncol* 2000, **18**, 4–11.
29. Picci P, Rougraff BT, Bacci G, *et al*. Prognostic significance of histopathologic response to chemotherapy in nonmetastatic Ewing's sarcoma of the extremities. *J Clin Oncol* 1993, **11**, 1763–1769.
30. Ferrari S, Bertoni F, Mercuri M, *et al*. Predictive factors of disease-free survival for non-metastatic osteosarcoma of the extremity: an analysis of 303 patients treated at the Rizzoli Institute. *Ann Oncol* 2001, **12**, 1145–1150.
31. Bieling P, Rehan N, Winkler P, *et al*. Tumour size and prognosis in aggressively treated osteosarcoma. *J Clin Oncol* 1996, **14**, 848–858.
32. Benini S, Baldini N, Manara MC, *et al*. Redundancy of autocrine loops in human osteosarcoma cells. *Int J Cancer* 1999, **80**, 581–588.
33. Scotlandi K, Manara MC, Strammiello R, *et al*. C-kit receptor expression in Ewing's sarcoma: lack of prognostic value but therapeutic targeting opportunities in appropriate conditions. *J Clin Oncol* 2003, **21**, 1952–1960.
34. Scotlandi K, Avnet S, Benini S, *et al*. Expression of an IGF-I receptor dominant negative mutant induces apoptosis, inhibits tumourigenesis and enhances chemosensitivity in Ewing's sarcoma cells. *Int J Cancer* 2002, **101**, 11–16.
35. Scotlandi K, Maini C, Manara MC, *et al*. Effectiveness of insulin-like growth factor I receptor antisense strategy against Ewing's sarcoma cells. *Cancer Gene Ther* 2002, **9**, 296–307.
36. Hughes DPM, Thomas DG, Giordano TJ, *et al*. Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. *Cancer Res* 2004, **64**, 2047–2053.
37. Cobleigh MA, Vogel CL, Tripathy D, *et al*. Multinational study of the efficacy and safety of humanised anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999, **17**, 2639–2648.
38. Menard S, Pupa SM, Campiglio M, *et al*. Biologic and therapeutic role of HER2 in cancer. *Oncogene* 2003, **22**, 6570–6578.
39. Lu Y, Zi X, Zhao Y, *et al*. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst* 2001, **93**, 1852–1857.
40. Lu Y, Zi X, Pollak M. Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. *Int J Cancer* 2004, **108**, 334–341.
41. Scotlandi K, Benini S, Nanni P, *et al*. Blockage of insulin-like growth factor-I receptor inhibits the growth of Ewing's sarcoma in athymic mice. *Cancer Res* 1998, **58**, 4127–4131.
42. Press MF, Hung G, Godolphin W, *et al*. *Cancer Res* 1994, **54**, 2771–2777.
43. Chan HS, Ling V. Anti-P-glycoprotein antibody C219 cross-reactivity with c-erbB2 protein: diagnostic and clinical implications. *J Natl Cancer Inst* 1997, **89**, 1473–1476.
44. Roessner A, Ueda Y, Bockhorn-Dworniczak B, *et al*. Prognostic implication of immunodetection of P glycoprotein in Ewing's sarcoma. *J Cancer Res Clin Oncol* 1993, **119**, 185–189.
45. Hijazi YM, Axiotis CA, Navarro S, *et al*. Immunohistochemical detection of P-glycoprotein in Ewing's sarcoma and peripheral primitive neuroectodermal tumours before and after chemotherapy. *Am J Clin Pathol* 1994, **102**, 61–67.
46. Perri T, Fogel M, Mor S, *et al*. Effect of P-glycoprotein expression on outcome in the Ewing family of tumours. *Pediatr Hematol Oncol* 2001, **18**, 325–334.
47. Slamon DJ, Leyland-Jones B, Shak S, *et al*. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *New Engl J Med* 2001, **344**, 783–792.
48. Piccart M. Closing remarks and treatment guidelines. *Eur J Cancer* 2001, **37**, S30–S33.