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ORIGINAL PAPER

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Clinical significance of the quantitative assessment of the cytosolic concentration of HER-2/neu protein in breast cancer by immunoenzymatic assay (ELISA)

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Abstract Purpose: Retrospective analysis to assess the prognostic and predictive value of HER-2/ *neu* expression in breast tumors, quantified by enzyme immunoassay (ELISA).*Methods:* Quantification of HER-2/neu was performed on cytosolic extracts from 914 cases of primary invasive breast carcinomas. Relapse-free and overall survival data were available from 889 patients. The prognostic value of HER-2/neu levels was assessed considering them as a continuous, dichotomic or quartile variable.*Results:* Cytosolic HER-2/neu levels ranged widely in breast carcinomas (median: 746.5 NHU/mg; range: 2.8–80,000 NHU/mg protein). HER-2/neu protein levels were significantly higher in either moderately or poorly differentiated tumors, as well as in those showing a ductal histological type, aneuploidy or a high S-phase fraction. There was a significant and positive association between cytosolic and membranous HER-2/neu levels ($n = 162$, r sub S = 0.53; $P < 0.0001$). In addition, cytosolic HER-2/neu level correlated weakly with progesterone receptors but not with estrogen receptors. Elevated cytosolic HER-2/neu levels (≥1,400 NHU/mg protein) were associated with a high probability of both shortened relapse-free survival and overall survival. This same cut-off value was obtained when we divided the overall group of patients in a training set. However, this HER-2/neu value did not achieve any statistical significance in a validation set used to make sure that the cut-off was correct. Nevertheless, when we divided the obtained data into three different groups with respect to the quartile values (Q) of the intratumoral oncoprotein levels ($\leq Q_1$ vs Q_1-Q_2 vs $> Q_3$), we observed that patients with either low HER-2/ *neu* levels ($\leq Q_1$) or high HER-2/neu levels ($> Q_3$) had shorter both relapse-free survival and overall survival curves than those patients with intermediate HER-2/neu levels. On the other hand, high HER-2/neu levels predicted a poor response to adjuvant chemotherapy but not to adjuvant hormonal therapy with tamoxifen.*Conclusions:* The results of the present investigation indicate that by quantitatively determining the content of HER-2/neu oncoprotein, groups of high-risk breast cancer patients could be identified, for a more effective clinical management.

Keywords c-erbB-2 · Prognosis · ELISA · Breast cancer · Her-2/neu

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Introduction

The proto-oncogene HER-2/neu, also referred to as c-erbB-2, is localized in chromosome 17q (Popescu et al. 1989) and encodes a transmembrane glycoprotein receptor of 185 kDa with intrinsic tyrosine kinase activity (King et al. 1985). Although the exact ligands for this receptor are not well defined yet, the oncoprotein

is thought to function as a growth factor receptor and seems to be involved in cellular differentiation, adhesion, and motility. In addition, HER-2/neu is the preferred co-receptor to form dimers with the epidermal growth factor receptor (EGFR, or HER-1), with HER-3 and HER-4. The heterodimers between HER-2/neu and these receptors show a greater capacity for translating mitogenic signals than the homodimers, being synergetic for cellular transformation (Pinkas-Kramarski et al. 1996; Graus-Porta et al. 1997).

Approximately 25–30% of invasive female breast carcinomas overexpress HER-2/neu (Slamon et al. 1987). In 90–95% of these cases, overexpression is a direct result of gene amplification (Pauletti et al. 1996). In some clinical studies, overexpression of the HER-2/neu gene has been associated with a number of adverse prognostic factors, including absence of estrogen receptors (ER) and/or progesterone receptors (PgR), aggressive histological subtypes, high histological grade, nuclear atypia and young age (Berger et al. 1988; Wright et al. 1989; Hoff et al. 2002; Konecny et al. 2003; Sapino et al. 2003; Varga et al. 2004; Tsuda et al. 1990). Likewise, the use of HER-2/neu as a prognostic factor in breast cancer has followed the original work by Slamon and coworkers (Slamon et al. 1987). Since then, several authors have reported a significant relation between the oncogene overexpression in primary tumors and a poor outcome in breast cancer patients (Rilke et al. 1991; Andrulis et al. 1998; Yamauchi et al. 2001). HER-2/neu status in breast cancer is also potentially useful for predicting the response to adjuvant therapy. Thus, retrospective analyses have demonstrated that there is a greater probability of tamoxifen resistance in patients overexpressing HER-2/neu (Wright et al. 1992; Borg et al. 1994; Carlomagno et al. 1996; Houston et al. 1999). Likewise, HER-2/neu overexpression has been reported to serve as a marker for resistance to adjuvant ciclophosphamide, metrothexate and 5-fluoruracil (CMF) (Allred et al. 1992; Gusterson et al. 1992; Stal et al. 1995; Pegram et al. 1997) as well as for anthracycline and taxanes sensibility in breast cancer (Muss et al. 1994; Paik et al. 1998, 2000; Petit et al. 2001; Moliterni et al. 2003; Stearns et al. 2003). On the other hand, overexpression of HER-2/neu on the surface of a tumor cell provides a potential target for the genetically engineered anti-HER-2/neu monoclonal antibody (Mab) trastuzumab (Hudziak et al. 1989). This antibody has been found to inhibit the proliferation of human breast cancer cells overexpressing HER-2/neu, both in vitro and in vivo. Likewise, a recent meta-analysis has suggested that amplification and/or overexpression of HER-2/neu in patients with metastatic breast cancer is a strong predictive factor for the response to adjuvant therapy with trastuzumab, either alone or in combination with cytotoxic agents such as taxanes and anthracyclines (Slamon et al. 2001).

In summary, we believe there could be three the principal reasons for the determination of HER-2/neu status in breast cancer patients: (a) to establish prognosis, (b) to predict response to adjuvant chemotherapy and endocrine therapy, and (c) to select patients for trastuzumab immunotherapy. Based on these concepts, determination of HER-2/neu status is now an integral part of the clinical-pathological workup in breast cancer. Currently, immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) are the most widely used techniques to detect HER-2/neu expression because both of them are ideally suited for routine and archival paraffin-embedded tissue, evaluating them by direct visualization of tumor cells. Screening by IHC and confirming indeterminate results by FISH testing has been advocated by many investigators (Kakar et al. 2000; Lebeau et al. 2001; Bilous et al. 2003). Nevertheless, these semiquantitative methods are subject to differences in methodology between laboratories, variability in operator interpretation, and disparity among the reagents utilized. Although FISH technology is reproducible, its major drawback is that the FISH equipment is expensive and not widely available in diagnostic pathology laboratories. In addition it is remarkable that despite all clinical research focused on this oncogenic receptor system, approximately 80% of patients with HER-2/neu-overexpressing breast cancer do not respond to trastuzumab (Pegram et al. 1999; Benz and Tripathy 2000), the prognostic impact of HER-2/neu in breast cancer is considered to be weak or poor, and present data are mostly inconsistent (Yamauchi et al. 2001).

HER-2/neu expression quantification by enzyme-linked immunosorbent assay (ELISA) is a method limited by the need of milligram quantities of fresh or frozen tumor sample, but it averts the potential antigen damage associated with fixation, embedding, and uncontrolled storage, yielding a highly reproductive continuous value obtained on the instrument readout (units per milligram of tumor protein). In addition, recent studies suggest that ELISA-based measurement of HER-2/neu protein concentration in breast cancer tissue extracts correlates with tumor aggressiveness and poor outcome (Eppenberger-Castori et al. 2001; Bohn et al. 2002; Konecny et al. 2003, 2004).

In the present work, we retrospectively investigate the potential clinical value of the HER-2/neu oncoprotein, quantitatively determined by using ELISA on cytosolic samples of primary tumors from a large series of breast cancer patients.

Materials and methods

Patient characteristics and tissue specimen handling

This study comprised 914 consecutive women with a histologically confirmed diagnosis of invasive breast cancer, who were treated at Hospital de Jove (Gijón, Spain) and at Hospital Central de Asturias (Oviedo, Spain), between 1990 and 2002. The mean age was 59.6 ± 13.4 years (range, 30–92 years). None of them

had undergone any neoadjuvant therapy nor shown evidence of any other malignant tumor at the time of diagnosis. Patients' characteristics with respect to age, menopausal status, and clinical tumoral stage are listed in Table 1. Histological grade was determined according to criteria reported by Bloom and Richardson (Bloom and Richardson 1957), whereas nodal status was assessed histopathologically.

Patients underwent either a modified radical mastectomy or a partial mastectomy with axillary lymphadenectomy. Postoperative radiotherapy was given to 119 patients (13.4%). Although the criteria for systemic adjuvant therapy varied somewhat during the study period, in the majority of cases they were as follows: (1) node-negative patients with ER and/or PgR-positive tumors received tamoxifen (20 mg per day during 5 years); (2) node-negative patients with ER and PgR negative tumors received six cycles of intravenous CMF every 3 weeks, if their tumors were either larger than 1 cm, moderately or poorly differentiated, or if the patients were younger than 35 years old; (3) node-positive patients received six cycles of intravenous (5-fluorouracil, epirubicin and cyclophosphamide FEC) every 3 weeks, plus sequential tamoxifen if they had ER and/or PgR-positive tumors. Overall, 265 patients received chemotherapy, 307 patients received tamoxifen, and 125 patients received both types of systemic therapy.

All patients were followed for disease recurrence and survival status by clinical and biological studies every 3 months for the first 2 years and then yearly. Radio-

Table 1 Tumoral HER-2/neu cytosolic levels in 914 breast carcinomas: correlation with different clinicopathological parameters

Patient and tumor characteristics	HER-2/neu (NHU/mg protein)					
	N°	Median	Range	*P*	> 746.5(%)	*P*
Total	914	746.5	2.8–80,000	–	457(50)	–
Age (years)				0.7		0.89
≤ 60	453	751	2.8–31,500		228(50.3)	
> 60	461	738	6.3–80,000		229(49.7)	
Menopausal status				0.6		0.61
Premenopausal	274	721.5	38.8–31,500		133(48.5)	
Postmenopausal	640	765	2.8–80,000		324(50.6)	
Size				0.3		0.61
T1	368	785	2.8–31,500		191(51.9)	
T2	391	738	15.7–37,209		194(49.6)	
T3	75	713	49–80,000		37(49.3)	
T4	80	608	49–23,789		35(43.8)	
Nodal status				0.16		0.15
N(−)	523	723	2.8–49,881		251(47.9)	
N(+)	383	838	15.7–80,000		203(53.0)	
Unknown	8	–	–		–	
Metastasis				0.5		0.6
M0	889	747	2.6–80,000		446(50.2)	
M1	25	623	49–23,789		11(44)	
Histological grade				0.001		0.0001
Well dif.	223	607	15.7–37,209		88(39.5)	
Mod. dif.	420	868.7	2.8–49,881		236(56.2)	
Poorly dif.	192	739.5	49–80,000		96(50)	
Unknown	79					
Histological type				0.02		0.028
Ductal	808	779	2.8–80,000		413(51.1)	
Lobular	63	715	6.3–5,753		31(49.2)	
Others	43	522	36.4–37,209		13(30.2)	
ER				0.65		0.94
Negative	406	743	2.8–80,000		202(49.8)	
Positive	506	749	15.7–30,329		254(50.2)	
Unknown	2	–	–		–	
PgR				0.09		0.10
Negative	476	814	2.8–80,000		252(52.9)	
Positive	392	713	36.4–14,016		185(47.2)	
Unknown	46	–	–		–	
Ploidy				0.001		0.03
Diploid	208	657.0	28.3–20,000		94(45.1)	
Aneuploid	336	839.5	2.8–80,000		184(54.7)	
Unknown	370	–	–		–	
Phase S				0.008		0.01
< 7.7	276	699.5	28.3–37,209		124(45.6)	
> 7.7	268	874.0	2.8–80,000		149(56.2)	
Unknown	370	–	–		–	

logical studies were performed either yearly or when considered necessary. The median follow-up period was 54 months (range, 1–168 months). The end-point was relapse or disease-specific survival. The median follow-up period in surviving patients was 60 months. Two hundred and thirty out of the 889 patients developed tumor recurrence, and 122 of them died of it.

Invasive breast carcinoma tissue samples were obtained at the time of surgery. Immediately after surgical resection, samples were processed for pathological examination while the remainder tissue was divided in aliquots, approximately 100 mg of the tissue was snap-frozen after removal of fat and connective tissue and stored in liquid nitrogen pending biochemical studies. Tissue samples were obtained with prior informed consent from the patients.

Immunohistochemical staining

$P185^{HER2}$ overexpression was assessed using the Dako Corporation (Carpinteria, CA, USA) HercepTest kit which uses a polyclonal antibody. It was chosen for its high affinity for the HER2 antigen in formalin-fixed, paraffin-embeded tissues. Following the manufacturer's guidelines, 5 mm sections were prepared and mounted on silanized slides (Dako Corp). After routine elimination of the paraffin and rehydraion, the slides were treated with an epitope retrieval solution for 20 min. After cooling to room temperature, the slides were treated with an epitope retrieval solution for 20 min, and this solution was then discarded and replaced by a wash buffer. Excess buffer was removed from each slide and a peroxidase-blocking reagent was applied for 5 min. The excess blocking reagent was decanted, and the slides were placed in a fresh wash buffer bath. The primary antibody (affinity purified rabbit anti-human HER2/neu) was applied for 30 min. Excess reagents were then applied for 30 min. After a final wash, tissue sections were treated with 3,3′-diamino-benzidine tetrahydrochloride (DAB) chromogen for 10 min. The slides were then rinsed in tap water, counterstained with hematoxylin, rinsed again in tap water, dehydrated, cleared, and coverslipped. Negative and positive controls were reviewed by two pathologists, who were unaware of the tumor $p185^{HER2}$ content. Each slide was scored according to the following scale:

1. 0, no staining.
2. 1+, incomplete membrane staining. These cases were easily confused with 0 staining at low power: Examination under high power revealed faint (usually focal) staining with a discernible but incomplete plasma membrane pattern.
3. 2+, complete membrane staining in 10% of the cells. Tissue staining in this type of case was recognized at low power, but examination under high power was necessary to identify a complete, unequivocal membranous staining pattern.
4. 3+, strong, complete plasma membrane pattern in the majority of cells, which could be appreciated at low power.

Tissue processing and assays

The specimens obtained from neoplastic tissues were pulverized with a microdismembrator (Braun Biotech International, Melsungen, Germany) at −70°C and homogenized in Tris–hydrochloride buffer (10 mM of Tris, 1.5 mM of EDTA, 10% glycerol, 0.1% of monothioglycerol). Homogenates, kept at 4°C, were then centrifuged at low speed (800 g for 10 min, at 4°C), and the supernatant was ultracentrifuged at 100,000 *g* for 60 min, at 4°C. We obtained in this way a supernatant containing the cytosol and a precipitate with the cell membranes. The membrane pellets were resuspended in Tris buffer and stored at −70°C until further analysis. Then the membrane pellets were homogenized in a glass-Teflon (E.I. du Pont de Nemours and company, Wilmington, USA) potter. The membrane samples were treated with Antigen Extraction Agent, used in sample preparation for the extraction of c-erbB-2 from membrane following the protocol included in the commercial kit.

HER-2/neu assay

The oncoprotein p185 was analyzed in membranous samples by an ELISA (Oncogene Research Products, Boston, MA, USA) that uses a mouse Mab for the antigen capture and a rabbit polyclonal serum for its detection, both binding specifically to the extracellular domain of the molecule. The analytical sensitivity of the method was 10 NHU/ml. The intra-assay and inter-assay coefficient of variations for concentrations of 33 and 104 NHU/ml of p185 were 4.3% and 2.4% ($n=16$) and 12.8% and 9% ($n=19$), respectively. The results were expressed as NHU/mg of protein homogenate (400 NHU/mg of protein correspond to 1 ng/mg of protein). The quantification of the oncoprotein was achieved by the construction of a standard curve using known concentrations of c-erbB-2 (provided by the commercial kit). By comparing the absorbance obtained from samples containing an unknown amount of c-erbB-2 with that from the standards, the concentrations of c-erbB-2 were determined.

Samples found to contain greater than 1,200 NHU/mg of protein homogenate (i.e., outside the range of the standard curve) were diluted with sample diluents, so that the c-erbB-2 concentration would fall within the range of the standard curve, and assayed again. Using this protocol, dilutions ranged from 1:10 to 1:1,000, thus being determined empirically. The protein content was quantified by the Bradford method (Bradford 1976).

Flow cytometry

DNA content was evaluated by flow cytometry in 544 tumors (Bectron Dickinson, San José, CA, USA), on nuclei stained with propidium iodide. DNA ploidy was expressed as DNA Index. Proliferative activity was expressed as the fraction of cells in the "S" phase of the cell cycle and calculated with the CellFit software program (Bectron Dickinson), according to the DNA Cytometry Consensus Conference recommendations (Hedley et al. 1993). Median S-phase fraction value was used as the cut-off point. Tumors were divided into those with a high or a low S-phase fraction.

Hormone receptor assays

ER and PgR receptor measurements were performed on cytosol extracts by using a solid phase enzyme immunoassay based on the "sandwich" principle (ER-EIA and PgR-EIA Monoclonal from Abbot Laboratories, Diagnostics Division, Wiesbaden, Germany). ER and PgR values were expressed as fentomols per milligram of protein. Protein concentration was quantified according to the described Bradford method (Hedley et al. 1993). For data analysis, a value higher than 10 fmol/mg total protein was considered as positive for ER and PgR.

Statistical analysis

After analyzing the distribution of HER-2/neu values by the Kolmogorov-Smirnov test, non-parametric rank methods were used. HER-2/neu content was expressed as median (range). Patients were subdivided into groups based on different clinical and pathological parameters. Comparison of the HER-2/neu content between groups was made with the Mann-Whitney and Kruskal-Wallis tests. Correlations between continuous variables were calculated by the Spearman test. Differences in percentages were calculated with the chi-square test. Probabilities of survival were calculated with the Kaplan-Meier method. Differences between curves were evaluated with the log rank test. The Cox's regression hazard proportional model was also used to examine several combinations and interactions of different prognostic factors in a multivariate analysis. In the multivariate analysis we included only parameters that achieve statistical significance for relapse-free survival or overall survival in the log rank test. The SPSS 11.5 program was used for all calculations. Statistical significance was considered at 5% probability level ($P < 0.05$).

Results

There was a wide range of cytosolic HER-2/neu protein levels among the breast carcinoma samples studied (2.8–80,000 NHU/mg protein; median: 746.5). The distribution of tumoral HER-2/neu protein levels is represented in Fig. 1. Table 1 shows the distribution of intratumoral HER-2/neu protein levels in relation to patient and tumor characteristics including age, menopausal status, tumor size, axillary node involvement, histological type, histological grade, ER and PgR status, ploidy and S-phase fraction. Statistical analysis showed that HER-2/neu protein levels were significantly lower in well-differentiated tumors than in moderately or poorly differentiated tumors ($P = 0.001$), as well as in those of ductal histology than any other histological types ($P = 0.002$). Likewise, HER-2/neu protein levels were also significantly higher in aneuploid than in diploid tumors, as well as in tumors with a high S-phase fraction than in those with a low S-phase fraction ($P = 0.001$ and $P = 0.008$, respectively). However, statistical analysis demonstrated that there was no significant relationship between HER-2/neu protein levels and age, menopausal status, tumor size, nodal status or ER and PgR status (Table 1). Nevertheless, HER-2/neu protein levels showed a weak but negative correlation with intratumoral PgR content in the overall group of patients ($n = 912$; r sub S $= -0.07$; $P = 0.03$) as well as in the subgroup of patients with ER-positive tumors ($n = 506$; r sub S $= -0.1$; $P = 0.025$). On the other hand, previous studies

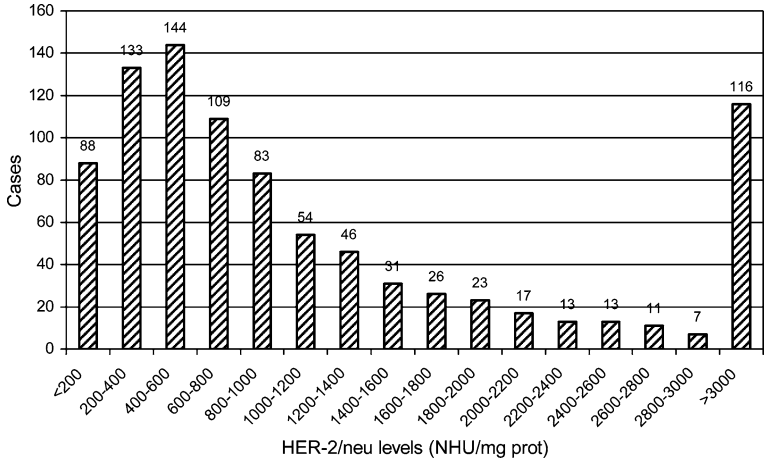


Fig. 1 Distribution of HER-2/neu cytosolic levels in 914 patients with breast carcinoma

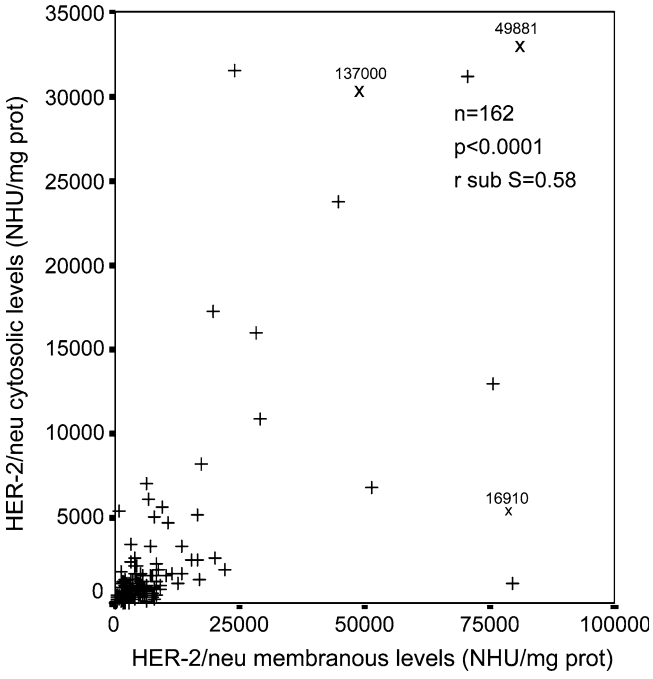


Fig. 2 Correlation between HER2-/ *neu* levels in membranous and in cytosolic tumoral samples

had pointed out the prognostic importance of membranous HER-2/neu levels measured by ELISA in breast cancer (Eppenberger-Castori et al. 2001; el-Ahmady et al. 2002). We therefore analyzed also the possible relationship between membranous and cytosolic levels of the oncoprotein in samples from a series of 162 breast carcinomas. In these 162 cases, membranous HER-2/neu protein levels (66.4–16,910 NHU/mg protein; median: 4,370) were significantly ($P < 0.0001$) higher than those found in the paired cytosolic samples (28.9–49,881; median: 923). Our study also demonstrated a significant and positive correlation between the HER-2/neu levels of these paired sets of tumoral samples ($n = 162$; r sub S = 0.53; $P < 0.0001$) (Fig. 2). Likewise, our results showed a significant and positive relationship between cytosolic levels of HER-2/neu and the staining values obtained by immunohistochemical assays in 77 tumors (Table 2).

These findings suggested a relationship between high intratumoral HER-2/neu levels and an unfavorable prognosis in breast cancer. To examine this hypothesis, the potential relationship between tumoral HER-2/neu protein levels and both relapse-free survival and overall survival were evaluated in the 889 patients without distant metastasis at the time of the initial diagnosis included in the present study. Firstly, we examined the possible prognostic value of the cytosolic HER-2/neu levels as a continuous variable. Our results showed a significant and positive association between the oncoprotein levels and relapse-free survival ($\chi^2 = 6.2$; $P = 0.014$), but not with overall survival ($\chi^2 = 2.4$; $P = 0.11$). Successively, we examined all possible HER-2/neu values as cut-off points for predicting relapse-free survival. As depicted in Fig. 3, our results demonstrated a continuous association between HER-2/neu values from 1,150 to 1,700 NHU/mg protein and relapse-free survival ($P < 0.05$). This analysis led us to define a value of HER-2/neu of 1,400 NHU/mg protein as the optimal cut-off ($\chi^2 = 6.3$; $P = 0.01$) giving us the possibility to identify a subgroup of 257 patients (28.1%) showing high HER-2/neu levels and a high risk of tumor recurrence that was also associated with overall survival ($\chi^2 = 7.3$; $P = 0.006$). Figure 4a,b shows both relapse-free survival and overall survival curves, respectively, considering the already mentioned cut-off value. This same value was the optimal cut-off for predicting the overall survival ($P = 0.047$) when we divided the patient group in a training set, which was randomly selected including the 66% of the data (the remaining data actuated as the validation test). However, this HER-2/neu value did not achieve statistical significance in a validation set to ensure that the cut-off was correct. Nevertheless, considering previous data regarding the non-monotone relationship between HER-2/neu tumoral levels by ELISA and prognosis in breast cancer patients (Dittadi et al. 1997; Koscielny et al. 1998), we divided all the data into three different groups with respect to the quartile values (Q) of the oncoprotein levels ($\leq Q_1$ vs Q_1-Q_2 vs $> Q_3$). As depicted in Fig. 5a,b, we observed that patients with either low HER-2/neu levels ($\leq Q_1$) or high HER-2/neu levels ($> Q_3$) showed both shorter relapse-free survival and overall survival curves than those patients with intermediate values (Q_1-Q_2) ($P = 0.08$ and $P = 0.01$, respectively).

Considering the possible influence of HER-2/neu status in predicting response to systemic treatment, survival analyses were also performed separately on the different subgroups of patients stratified according to the type of adjuvant systemic therapy received. In each one of these subgroups, patients were dichotomized into two different groups with regard to the optimal cut-off value of intratumoral HER-2/neu levels (1,400 NHU/mg protein). As it can be seen in Table 3, intratumoral HER-2/neu levels were not significantly associated with a higher probability of both relapse-free survival and overall survival in the subgroup of patients treated only with tamoxifen or in those patients without any type of systemic adjuvant treatment. However, high HER-2/neu levels were in correlation with shortened both relapse-free survival and overall survival in patients who received only chemotherapy (Fig. 6a,b) and in those

Table 2 Relationship between p^{185} immunostaining values and p^{185} levels by ELISA

p^{185} Staining	N	ELISA levels (NHU/mg protein)		
		Median	Range	
0/+	41	612	2.8–25,55	$P = 0.004$
++	23	811	36.4–23,789	
+++	13	2851	592–49,881	

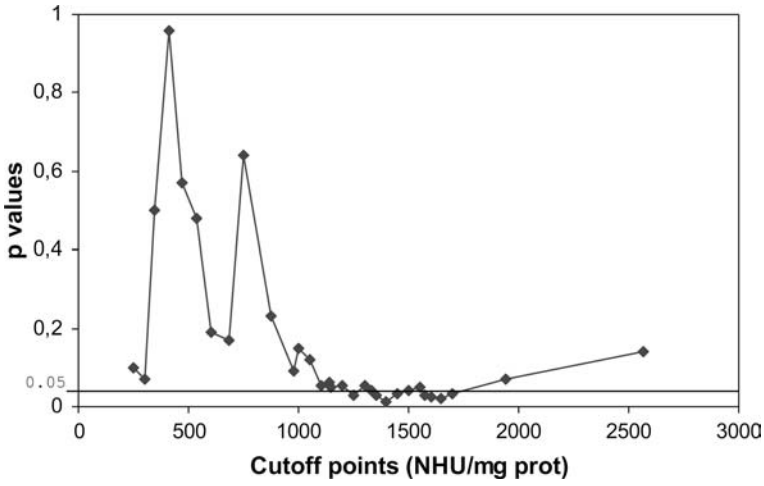


Fig. 3 Maximum likelihood determination of the cutoff value for the cytosolic HER-2/neu tumoral content (NHU/mg protein) to predict relapse-free survival in 889 patients with breast cancer. *P*-values obtained for each cutoff value are plotted against the value itself. Statistical significance is indicated by the horizontal line at the 0.05 level. Analyses lead to the definition of a HER-2/neu content of 1,400 NHU/mg protein as the optimal cutoff ($\chi^2 = 6.3$; $P = 0.012$)

treated with chemotherapy plus tamoxifen as adjuvant therapy (Table 3; Fig. 7a,b). On the other hand, we did not find any significant differences in both relapse-free and overall survival curves with regard to the adjuvant systemic treatment when patients were stratified in three groups according to their intratumoral HER-2/neu levels as indicated above ($\leq Q_1$ vs Q_1-Q_2 vs $> Q_3$) (data not shown).

Finally, multivariate analysis according to Cox model demonstrated that tumoral size, nodal status, ER status and HER-2/neu levels (as dichotomized in 1,400 NHU/mg protein) were significantly associated with both relapse survival and overall survival in all the patients. Likewise, histological grade was also associated with relapse-free survival (Table 4). Nevertheless, multivariate analysis confirmed that intermediate HER-2/neu levels (Q_1-Q_3) were significantly related with a more prolonged overall survival [RR = 0.65; CI (95%) = 0.43–0.9] compared with patients with low ($\leq Q_1$) or high ($> Q_3$) HER-2/neu intratumoral levels (P = 0.043).

Discussion

This is, to our knowledge, the largest clinical study evaluating the prognostic value of cytosolic HER-2/neu levels in breast carcinomas determined quantitatively by ELISA. Our results show a wide range of intratumoral oncoprotein levels, which seems to correspond to the biological heterogeneity of these tumors. Likewise, high HER-2/neu levels correlate with tumor parameters indicative of tumor aggressiveness and with a poor outcome. Nevertheless, the relationship between HER-2/neu levels and prognosis was non-monotone. Thus, we also found a subgroup of patients with low HER-2/neu levels having a short overall survival.

IHC and FISH are the most commonly employed methods to assess the HER-2/neu status in clinical samples of breast carcinomas. In this study, we chose ELISA technology because it is the only large scale testing method able to measure HER-2/neu protein expression levels in a quantitative fashion, this way being a more objective method of detecting the oncoprotein expression and perhaps being biologically a more realistic model as a continuous variable. Several authors have reported that ELISA-based measurement of HER-2/neu protein concentration in breast cancer tissue extracts correlates with immunohistochemical staining or gene amplification (Dittadi et al. 1993; Nugent et al. 1994; Piffanelli et al. 1996; Eppenberger-Castori et al. 2001; Muller et al. 2003). Similarly, in a prior report (Lamelas et al. 2003) as well as in the present study, we have found a significant and positive relationship between the IHC staining for HER-2/neu protein, using the HercepTest Kit (Dako Corp., Carpenteria, CA, USA), and the cytosolic oncoprotein

Table 3 Univariant analysis of the relationship between intratumoral HER-2/neu cytosolic levels and relapse-free and overall survival in patients with breast cancer, stratified according to the received adjuvant systemic therapy

Group	Patients	Relapse-free survival		Overall survival	
		HR* (CI)	p	HR* (CI)	p
Chemotherapy	265	1.5(1.02–2.4)	0.04	2.3(1.2–4.3)	0.007
Tamoxifen	307	1.2(0.6–2.1)	0.50	1.3(0.6–2.9)	0.4
Chemotherapy plus sequential Tamoxifen	125	2.3(1.2–4.3)	0.008	3.1(1.3–7)	0.009
No treatment	158	0.8(0.4–1.8)	0.7	0.6(0.1–2.0)	0.6

Hazard ratio
*HER-2/neu: < median vs > median

Table 4 Multivariate analysis of the association between HER-2/neu cytosolic levels, relapse-free and overall survival

Tumor characteristics	Relapse-free survival			Overall survival		
	RR	CI (95%)	*P*	RR	CI (95%)	*P*
Overall group						
Tumor size			0.001			0.001
T1	1	–		1	–	
T2	1.8	1.3–2.6		02.4	1.4–4	
T3	1.9	1.1–3.2		2.7	1.3–5.8	
T4	2.6	1.6–4.2		4.7	2.5–9.1	
Nodal status			0.001			0.001
Negative	1	–		1	–	
Positive	2.8	2.1–4		3	1.9–4.6	
Histological grade			0.031			–
Well dif.	1	–		–	–	
Mod. dif.	1.6	1.1–2.4		–	–	
Poorly dif.	1.7	1.0–2.6		–	–	
ER			0.005			0.024
Negative	1	–		1	–	
Positive	0.65	0.4–0.8		0.6	0.4–0.9	
NEU levels			0.021			0.019
< 1,400 NHU/mg prot	1	–		1	–	
≥1,400 NHU/mg prot	1.4	1.0–1.9		1.6	1.0–2.4	

RR relative risk, *CI* confiance interval

concentration quantified by ELISA in breast carcinomas. Nevertheless, it is important to emphasize that validating ELISA as an alternative method to IHC for the detection of HER-2/neu overexpression in clinical practice was not an objective of this study. In addition, although there are data indicating that measuring HER-2/neu by ELISA correlates appropriately with the results obtained by either ICH or FISH, it seems that different methodological approaches of measuring the oncoprotein are not completely equivalent (Dittadi et al. 1993; Piffanelli et al. 1996; Eppenberger-Castori et al. 2001; Lamelas et al. 2003; Muller et al. 2003). It has also been indicated that the observed discrepancies between in situ hybridization and ELISA measurements could be explained by the fact that the one single tissue section needed for FISH does not represent all the tumor material that is used for tissue homogenization in ELISA (Muller et al. 2003). Likewise, one consideration that points to the value of direct measurement of c-erbB-2 protein content is that its overexpression in human tumors may also occur without gene amplification, involving other mechanisms such as up-regulation of c-erbB-2 transcription (Tandon et al. 1989). A possible downside of the ELISA technique is the contamination of tissue homogenates of tumor sample with mixed cell type populations and/or non-cellular connective tissue composed largely of extracellular matrix proteins, which could be the cause of errors due to dilutional artifacts (Slamon et al. 1989; Press et al. 1993). In addition, the contamination with ductal cancer "in situ" (DCIS) (which has often a high HER-2/neu expression) could alter the HER-2/neu concentration determined in tissue extracts. Nevertheless, we consider that the large sample size in the present study allowed us to overcome some of the potential limitations introduced by stromal cell or DCIS contaminations in the ELISA.

Our results are in accordance with prior studies where HER-2/neu was also determined in cytosolic samples of breast carcinomas using ELISA, and reporting an association between the elevated concentration of the oncoprotein and different parameters indicative of tumor aggressiveness, such as ductal histology and poorly differentiated histological grades (Bohn et al. 2002), as well as with shortened relapse-free and/or overall survival (Nugent et al. 1992; Eissa et al. 1997; Bohn et al. 2002; Konecny et al. 2003, 2004). In addition, our results indicate that HER-2/neu cytosolic levels are significantly associated with aneuploidy and with higher S-phase fraction in breast carcinomas. Besides, it is also remarkable that previous studies have shown that high HER-2/neu levels in membranous samples of breast carcinomas, also evaluated by ELISA, correlated with a poor outcome (Eppenberger-Castori et al. 2001; el-Ahmady et al. 2002). With respect to these findings, in the present study we have demonstrated, for the first time to the best of our knowledge, that there is a positive and significant relationship between membranous and cytosolic HER-2/neu levels in breast carcinomas. Nevertheless, although this finding suggests a connection between these oncoprotein levels, the low correlation value found between both ($n = 162$; r sub $S = 0.528$; $P < 0.0001$) might reflect a different biological signification and/or a different clinical value. Further investigations will be necessary to elucidate this question.

An important issue on the quantitative measurement of biological factors in cancer is being able to define the

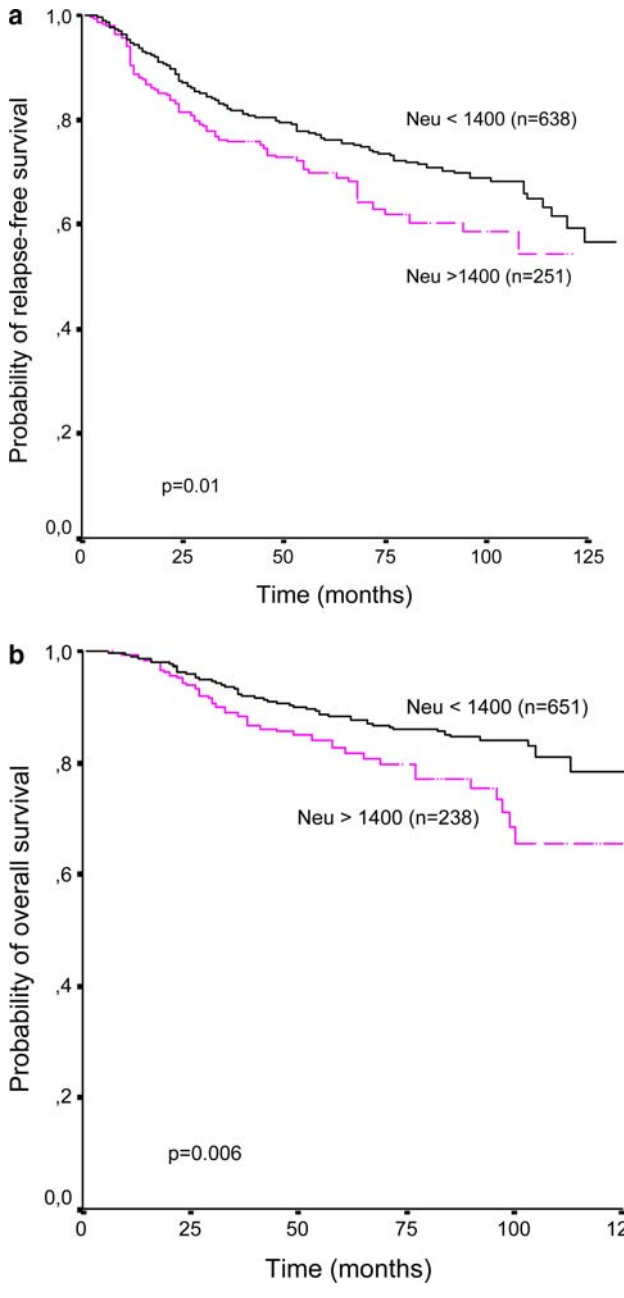


Fig. 4 Relapse-free survival (**a**) and overall survival (**b**) as a function of the cut-off value of HER-2/neu cytosolic levels in 889 patients with breast carcinoma

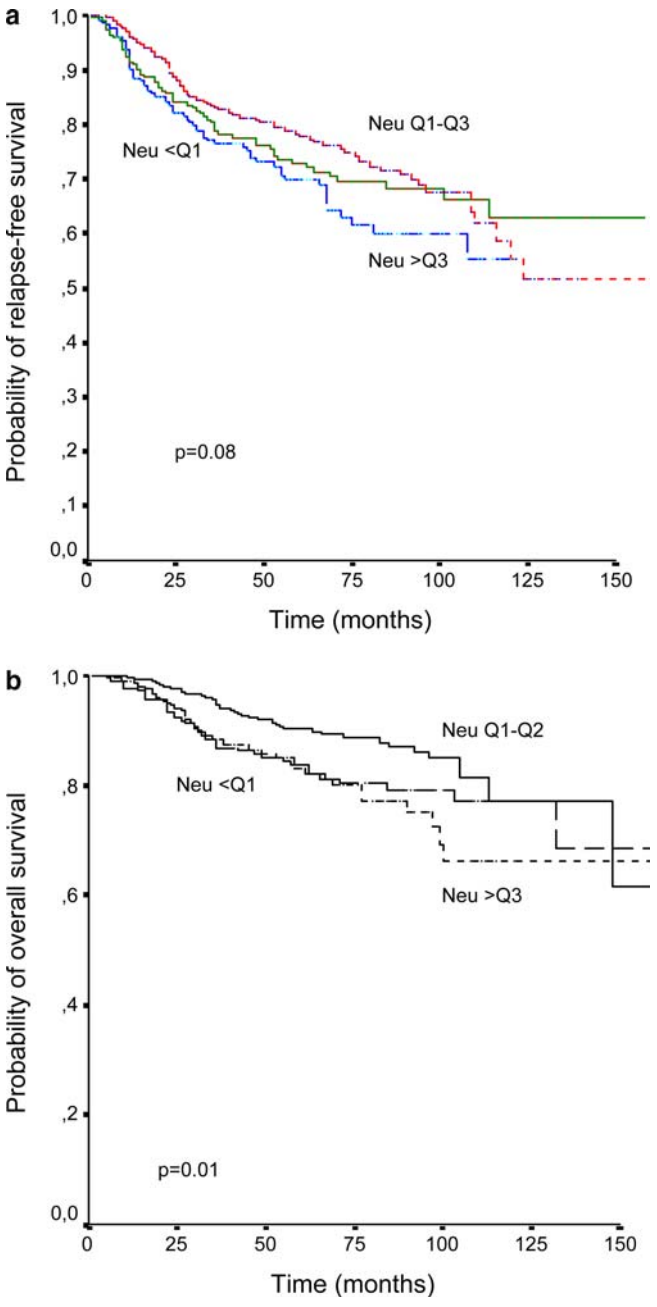
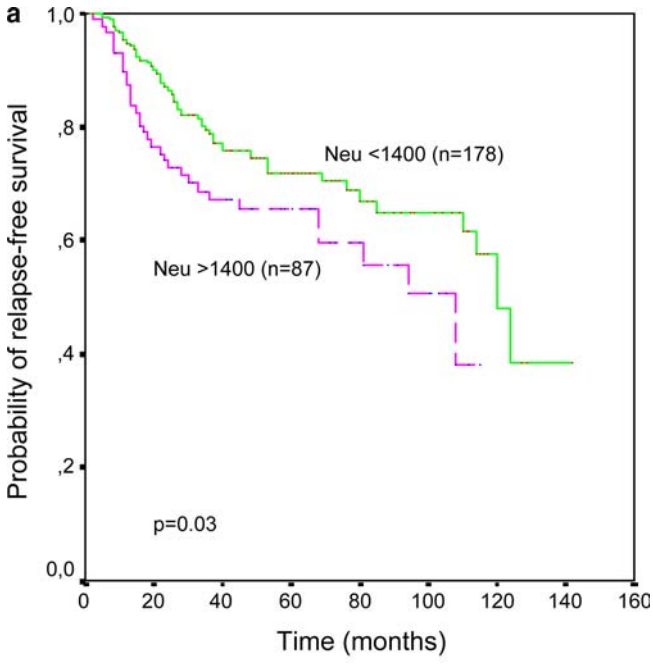


Fig. 5 Relapse-free survival (**a**) and overall survival (**b**) as a function of the quartile values of the HER-2/neu cytosolic levels in 889 patients with breast carcinoma

optimal cut-off value of clinical interest. In previous ELISA studies on tissue extract from breast carcinomas, this value was chosen in compliance with several criteria, such as validation according to the percentages of positives within the range of the cases observed by using gene amplification or overexpression of HER-2/neu in breast carcinomas (Valeron et al. 1997; Koscielny et al. 1998; Bohn et al. 2002) or in cell lines (Konecny et al. 2003; Konecny et al. 2004), defined as the highest level of expression in non-malignant tissue (Piffanelli et al. 1996), or as an intermodal value of a bimodal distribution of the oncoprotein levels (Eppenberger-Castori et al. 2001). In the present work, we have investigated the clinical interest of all the obtained values of HER-2/neu cytosolic levels. Our results show a significant value as continuous variable of these levels. However, by dichotomizing the variable we have not been able to identify an optimal cut-off value with a statistically independent value to predict both relapse-free and overall survival. Nevertheless, we have also evaluated the distribution in quartiles to study also the possible non-monotone relationship between HER-2/neu and outcome in breast cancer. Thus, we have observed that patients with either low HER-2/neu or high HER2/ *neu*



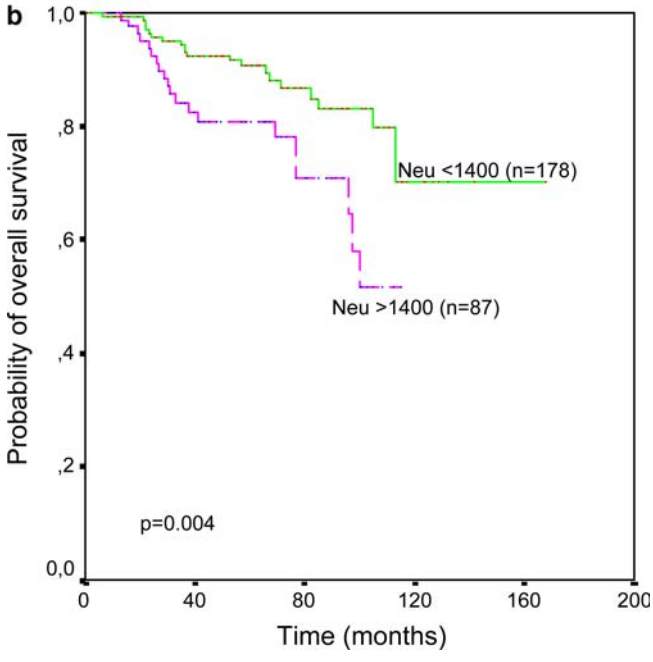
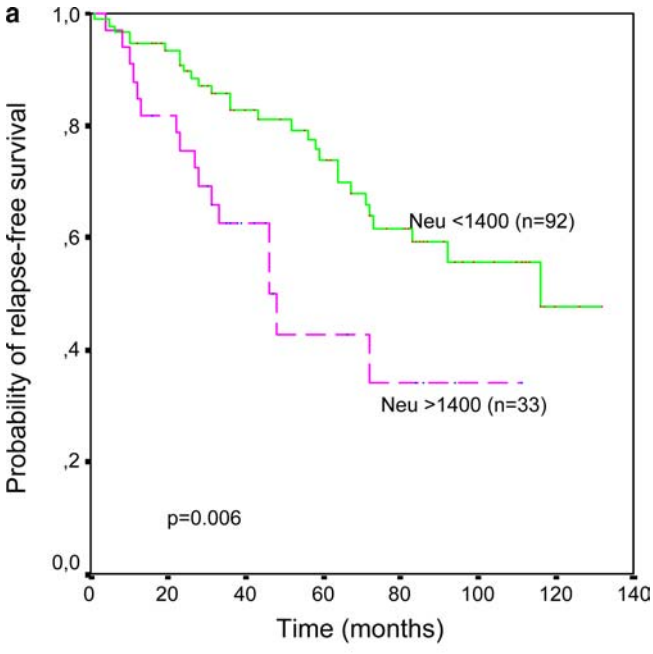


Fig. 6 Relapse-free survival (**a**) and overall survival (**b**) as function of the HER-2/neu cytosolic levels in 265 patients with breast carcinoma and chemotherapy as the single systemic adjuvant therapy



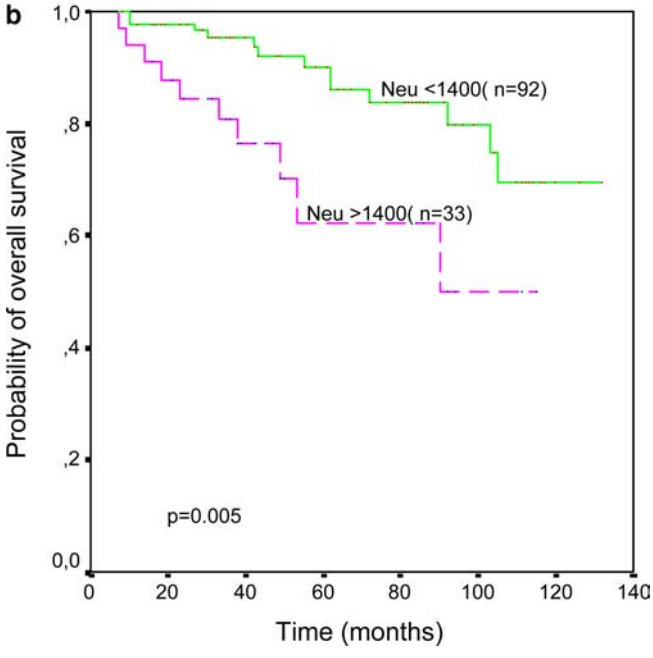


Fig. 7 Relapse-free survival (**a**) and overall survival (**b**) as function of the HER-2/neu cytosolic levels in 125 patients with breast carcinoma and treated with chemotherapy plus tamoxifen as systemic adjuvant therapy

levels had shorter overall survival than patients with intermediate intratumoral concentration of the oncoprotein. These findings, although somewhat surprising, coincide with those obtained by two other studies in which HER-2/neu levels were measured quantitatively using ELISA by a system similar to ours (Dittadi et al. 1997; Koscielny et al. 1998). It has been reported that the high concentration group corresponds closely to the positive samples detected by IHC and they could be expected to have a poorer prognosis. With regard to the finding of high-risk patients with very low HER-2/neu expression, it has been suggested that the progress to a more aggressive disease could imply selection against HER-2/neu over-expression or down-regulation due to a high binding activity (Hynes and Stern 1994). It has also been implied that an interaction between various oncogenes could explain this phenomenon, such that the absence of expression of one of them is counterbalanced by the overexpression of another in the context of all

deviations from the "normal" expression (Koscielny et al. 1998). Therefore, it is possible that at least some of the samples with low HER-2/neu expression correspond to tumors with an aggressive phenotype. Consequently, the use of a quantitative method seems to allow for a more complete identification of patients at risk, who are not necessarily represented only by those with a higher HER-2/neu expression.

Although the exact mechanism by which HER-2/neu regulates this more aggressive phenotypes is not fully understood, it has been shown that HER-2/neu overexpression is involved in cell proliferation and causes enhancement of cell migration in human breast cancer cells (Verbeek et al. 1998), which may confer a growth advantage in the early stages of breast carcinoma. Recent studies have also indicated that HER-2/neu receptors play an important role in the induction of angiogenesis (Yen et al. 2000; Izumi et al. 2002; Konecny et al. 2004). Another mechanism for which HER-2/neu overexpression may be implicated in tumor progression is by reducing endocrine responsiveness and promoting drug resistance. Accordingly, some authors have reported an inverse association between HER-2/neu and ER levels in breast carcinomas by using an ELISA, IHC or FISH (Zeillinger et al. 1989; Borg et al. 1990; Nugent et al. 1992; Marsigliante et al. 1993; Quenel et al. 1995; Tagliabue et al. 1999; Eppenberger-Castori et al. 2001; Konecny et al. 2003) . However, contrary to these previous studies, our results do not show an inverse relationship between HER-2/neu oncoprotein and ER levels (Konecny et al. 2003). Nevertheless, an interesting finding from our investigation is the significant and inverse, although weak, association between HER-2/neu and PgR levels. This observation may have arisen from the fact that PgR expression more accurately represents the functional status of hormone receptors in breast cancer than does the ER expression, because PgR expression is linked to a biologically active and functional ER (Fuqua et al. 1991; Wang et al. 1999). This observed inverse association between HER-2/neu and steroid hormone receptors status might explain the data from different retrospective analyses demonstrating that there is a greater probability of tamoxifen-resistance in patients overexpressing HER-2/neu (Borg et al. 1994; Houston et al. 1999). These clinical observations are consistent with preclinical data indicating there might be receptor crosstalk between the HER-2/neu and ER signal transduction pathways. Thus, there are cell line data showing that the introduction of additional HER-2/neu copies into MCF-7 cells and the corresponding increase in HER-2/neu expression leads to a reduction in the estrogen-binding capacity of the cells and in ER and in PgR transcripts (Pietras et al. 1995). Likewise, it has been shown that the activation of growth factor receptors such as Her-2/neu may result in a direct phosphorylation and activation of ER in an estrogen-independent manner, which may itself be an important mechanism for tamoxifen resistance (Smith 1998). However, although some reports have indicated that HER-2/neu overexpression reduces both response duration and survival duration in patients treated with adjuvant tamoxifen (Stal et al. 2000; De Placido et al. 2003; Osborne et al. 2003), our results are rather in accordance with other reports which have failed to observe this association in patients receiving tamoxifen as a single systemic adjuvant therapy (Berry et al. 2000; Love et al. 2003).

With respect to the value of HER-2/neu overexpression as a predictor of response to systemic adjuvant chemotherapy, although our study was not specifically conducted to evaluate the type and dosing of the chemotherapeutic agents, while these varied over the studied period, our results suggest a poorer response to this general type of adjuvant therapy in patients with high HER-2/neu intratumoral levels. The implications of HER-2/neu overexpression on the efficacy of adjuvant chemotherapy have also been controversial. There are retrospective studies of adjuvant chemotherapy with CMF or similar regimens indicating a poor prognosis in patients with HER-2/neu overexpressing tumors (Allred et al. 1992; Gusterson et al. 1992; Stal et al. 1995); whereas a more recent report comparing the efficacy of CMF relative to observation in HER-2/neu-positive and HER-2/neu-negative patients has not supported this finding (Miles et al. 1999). Likewise, several reports suggest an increased response to anthracycline-containing adjuvant chemotherapy in patients with HER-2/neu-positive tumors (Muss et al. 1994; Paik et al. 1998; Thor et al. 1998; Moliterni et al. 2003). Nevertheless, considering that in all of these reports HER-2/neu status was assessed by IHC assays, we might assume that the corresponding HER-2/neu values for predicting resistance/sensitivity to systemic adjuvant therapy may differ from those found in the present work. Hence, further specific studies are necessary to evaluate the possible clinical value of quantitative measurements of intratumoral oncoprotein levels in order to predict response to adjuvant therapies in patients with breast cancer.

On the other hand, the importance of HER-2/neu status as a predictor of tumor response to systemic therapy using the humanized HER-2/neu antibody trastuzumab (Herceptin) is evident (Slamon et al. 2001). At present, a combination of IHC and FISH or FISH alone is being used to evaluate HER-2/neu status, and these assays can be performed on archival formalin-fixed parafin embedded tissue should the clinical need arise (usually in patients developing metastasic brest cancer). Nevertheless, considering that an increasing number of centers in Europe and the US are preparing tissue extracts from primary breast cancer specimens for determination of urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) for prognostic and predictive purposes (Janicke F et al. 2001; Look et al. 2002), it has been suggested that these tissue extracts are available for the additional assessment of HER-2/neu status by an ELISA-based approach (Muller et al. 2003). Thus, as re-

gards anti-HER-2/neu therapy, future investigations could address the possible predictive value provided by this ELISA-based method.

In summary, our results suggest that by quantitatively determining the HER-2/neu oncoprotein by the use of ELISA, groups of patients with high-risk breast cancer could be identified.

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