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Tissue Expression and Serum Levels of the Oncoprotein HER-2/*neu* in 157 Primary Breast Tumours

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Abstract. *Background: We studied HER-2 expression in paired serum and tissue samples, in 157 selected cases from 701 consecutive primary breast cancer patients with pre-treatment HER-2 extracellular domain (ECD) ≥ 10 ng/ml, or < 10 ng/ml but showing a HER-2 ECD lead time before first metastasis. Patients and Methods: HER-2 ECD was measured by the Immuno 1 automated ELISA (Bayer). Tumour tissue was analysed by immunohistochemistry (IHC) with Dako A 0485 and CB 11 antibodies and scored with the Dako scoring system. Results: Mean HER-2 ECD was 12.48±7.08 ng/ml and 21/157 (13.4%) sera were ≥ 15 ng/ml (cut-off). Forty tumours (25.48%) showed both invasive and intraductal components, 3 (1.91%) were pure in situ carcinomas and 114 (72.61%) were pure invasive tumours. Elevated HER-2 ECD concentration was related only to pT (p=0.0008), histological grade (p=0.0465), presence of comedonecrosis (p=0.0123) or comedo-type carcinoma (p=0.041) and was unrelated to the presence of an intraductal component. HER-2 ECD was ≥ 15 ng/ml in 48% of Dako 3+ and 60% of CB 11 2+ and 3+ tumours. By logistic regression analysis, the significant parameters associated with HER-2 ECD concentration were pT (p=0.0038) and Dako 3+ scores (p=0.0005). In Dako 3+ or CB 11 2+3+ tumours, elevated mean HER-2 ECD concentrations were observed only when pT exceeded 28-30 mm (p=0.0062 and p=0.0036, respectively). Conclusion: In breast tumours, a threshold in size and HER-2 overexpression is necessary to observe elevated concentrations of HER-2 ECD at diagnosis. This information may be useful when the primary tumour is not available for IHC.*

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The HER-2 oncogene (HER-2/*neu* or c-erbB-2) is pivotal to the tumorigenesis and clinical behaviour of breast tumours. HER-2 overexpression/amplification is observed in 20-30% of primary breast tumours and is associated with tumour aggressiveness, metastatic disease and poor prognosis (1). The HER-2/*neu* oncogene encodes a 185 kDa transmembrane glycoprotein growth factor receptor, which is one of the four members of the epidermal growth factor receptor family. The HER-2 receptor comprises an extracellular ligand-binding region, a transmembrane domain and displays tyrosine kinase activity on its intracytoplasmic domain (2).

Overexpression of HER-2/*neu* in breast tumours is currently determined by immunohistochemistry (IHC) or by fluorescence *in situ* hybridization (FISH), which are currently the reference methods (3). The score obtained is used to determine patient eligibility for targeted therapy with the monoclonal antibody trastuzumab. However, these tests require access to cell or tissue samples (primary tumour or recurrence) and, thus, cannot provide clinicians with longitudinal assessments following removal of the primary tumour tissue. Immunohistochemical methods are dependent on the type of antibody used and on semi-quantitative evaluation by a pathologist. They show significant discordance in distinguishing 2+ and 3+ levels of HER-2/*neu* overexpression. FISH also yields semi-quantitative scores for relative DNA copy numbers (c-erbB-2 gene signals per nucleus or gene signals per chromosome 17), but is a specialised technique, not routinely available, which also displays some level of discrepancy (4, 5).

The extracellular domain (ECO) of the HER-2/*neu* receptor (HER-2 ECD) is present in the circulation and its concentration in cancer patients may be elevated (6). Recent reports indicate that measurements of HER-2 ECD show clinical utility in the monitoring of breast cancer patients and potentially provide a highly reproducible and straightforward alternative to morphological techniques (7). The relationships of baseline circulating HER-2 ECD levels with HER-2 overexpression in primary breast tumours have been little

studied. This work presents a comparison between pre-treatment HER-2 ECD concentration and HER-2 overexpression, evaluated by two different antibodies, in paired tumours from a series of 157 patients at the time-point of primary breast cancer.

Patients and Methods

Study patients. Aliquot portions of sera from breast cancer patients were collected prospectively and stored at –30˚C in our blood bank from 1.1.1992. We retrieved 701 consecutive new primary cases from January 1992 to 1st July 1994 at René-Huguenin Cancer Centre, France, and followed up thereafter for previous studies of HER-2 ECD at diagnosis and during monitoring (8 ,9). Sera obtained before primary treatment, surgery or neoadjuvant treatment were assayed for HER-2 ECD. From the 701 primary breast cancer patients, we selected all the cases having a pre-treatment HER-2 ECD concentration ≥10 ng/ml, without knowledge of their histological characteristics. The corresponding primary tumours were retrieved from archival blocks kept in the Pathology Department. Primary tumour tissue was available for 144/150 cases with HER-2 ECD ≥10 ng/ml. In addition, we analysed 13 other cases from the same series with pre-treatment HER-2 ECD below 10 ng/ml, but which showed a lead time for HER-2 ECD during monitoring.

HER-2 ECD assays. HER-2 ECD concentrations were measured with an automated HER-2/*neu* assay (Bayer Immuno 1, Bayer Diagnostics, Tarrytown, NY, USA) (10). All samples were analysed in duplicate. The Bayer Immuno-1 HER-2/*neu* assay is a magnetic particle separation enzyme immunoassay that uses two antibodies binding to independent sites on HER-2 ECD. Stably transfected mouse NIH 3T3 cells secreting a recombinant human HER-2/*neu* extracellular domain (ECD) are used in this assay to prepare calibrators and control sera. Quality control was ensured by assaying the three levels of control sera supplied with the kits in each series. Mean±SD and coefficient of variation (CV) for the controls were 15.8±0.74 ng/ml (CV 4.87%), 49.72±1.93 ng/ml (CV 3.88%) and 100.47±4.02 ng/ml (CV 4.00%), (n=33).

Pathological analysis of tumours. All the histological slides of the 157 tumours were analysed without knowledge of HER-2 ECD serum concentrations. Tumour histological size (pT) and detailed microscopic features were recorded regarding the invasive and intraductal components and the stroma (Tables II and III). Histopathological grading was performed according to the Scarff and Bloom (SBR) method (11), while grading of ductal carcinomas *in situ* (DCIS) and intraductal components followed the Holland grading (12). On the basis of the percentage of intraductal components, we grouped tumours into three categories: pure invasive carcinomas, mixed *in situ* and invasive carcinoma (tumours with an important intraductal component extending at distance from the tumour) and pure DCIS (13).

Immunohistochemical evaluation of HER-2 overexpression. For each case, a representative block of the tumour was selected. When tumours were bifocal, one block of each tumour was analysed to verify concordance between the tumours. Tumours were fixed in neutral buffered formalin and the IHC procedure followed the guidelines of the Groupe d' Evaluation des Facteurs Pronostiques par Immunohistochimie dans les Cancers du Sein (GEFPICS) and the United Kingdom guidelines (14,15). IHC staining was performed with an automated slide stainer (Autostainer, Dakocytomation, Glostrup, Denmark). We selected two widely used primary antibodies, a rabbit polyclonal (A 0485, Dako) and a mouse monoclonal antibody (mAb) (CB11, Zymed, San Francisco, USA) both recognizing the internal domain of HER-2 membrane receptor. Briefly, each block was cut at 4-5 μm thick, dried overnight in an oven, dewaxed in toluene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed during 40 min in a water bath set at 95-98˚C, followed by 20 min cooling. An in-house control slide, including 3 tumour fragments scored respectively 3+, 2+ and 0 validated by FISH, was added for staining quality control. In addition, we participate in the UK NEQAS (United Kingdom National External Quality Assessment) and AFAQAP (Association Française d' Assurance Qualité en Anatomie et Cytologie Pathologiques) external quality controls. The primary antibodies were incubated at 1/1000 dilution (Dako) and 1/400 (CB 11).The revelation was based on the streptavidin-biotin system (Dako LSAB) with 3-3' diaminobenzidine tetrahydrochloride as peroxidase substrate. Both the percentage of stained cells and intensity of staining were assessed. We used the Dako scoring system to score the staining obtained with both antibodies, following the UK and US recommendations (15-17). In the present work, microscopic analysis performed for each tumour included first the examination of the hematoxylin-eosin slide, then the HER-2 Dako staining, ending with the CB11 staining. With CB 11 mAb, immunostaining 2+ and 3+ were considered as overexpression according to Press *et al.* (18).

Statistical methods. Differences in the distribution of characteristics between patient subgroups were analysed by the Chi-square test or the Fisher's exact test. Differences between two independent groups were determined with the Mann-Whitney *U*-test. Kruskal-Wallis one-way analysis of variance was used to test for overall homogeneity and, in cases where the test was significant ($p<0.05$), pairwise comparisons were performed with the Mann-Whitney *U*-test. The difference between two continuous variables was tested by the Student's *t*-test. Agreement between staining scores of intraductal and invasive components was tested by using the weighted kappa statistic. The percent of stained cells was compared by the Spearman rank test. To assess the relative importance of the clinical, histological and immunohistochemical variables regarding baseline HER-2 ECD, a logistic regression analysis was performed in the subgroup of patients with pure invasive ductal carcinoma (cytoplasmic staining excluded) with baseline HER-2 ECD dichotomised according to the cut-off (15 ng/ml) as the dependent variable. The Hosmer-Lemeshow statistic was computed for the model and then compared to a Chi-square distribution with one degree of freedom. A large value for this statistic (hence small *p*-value) indicates a lack of fit of the model. The SAS statistical package (SAS Institute, Cary, NC, USA) was used for univariate and multivariate analyses and MedCalc software (Mariakerke, Belgium) was used to calculate the weighted kappa statistic and ROC (receiver operating characteristics) curves.

Results

The main clinico-biological characteristics of the subgroup selected on the basis of HER-2 ECD concentration are summarized in Table I (n=157). Several significant differences were noted when compared to the unselected counterpart (n=544): in the latter group, mean age (54.5±12.5 years) was

Table I. *Tumour and patient characteristics (n=157).*

Variables	Categories	Number of patients (%)
Age (years)	Mean±SD	59.5±12.3
menopause	no	45 (28.7%)
	yes	112 (71.3%)
T	0	4 (2.5%)
	1	33 (21.0%)
	2	98 (62.4%)
	3	13 (8.3%)
	4	9 (5.7%)
N	0	112 (71.3%)
	1	43 (27.4%)
	2	2 (1.3%)
M	0	157 (100%)
Inflammatory cancer	no	148 (94.3%)
	yes	9 (5.7%)
pT (mm)	Mean±SD	26.6±12.4
pN	Mean±SD	2.5±4.9
ER (cytosol) *	negative	45 (28.7%)
	positive	112 (71.3%)
PgR (cytosol)*	negative	61 (38.9%)
	positive	96 (61.1%)
Serum HER-2 ECD (ng/ml)	Mean±SD	12.48±7.08
	Median (range)	11.15 (6.30-82.00)

*threshold for positivity: > 10 fmoles/mg cytosol protein.

Table II. *Histopathological data. Part I : invasive component.*

Variables	Categories	Invasive and intraductal (n=40)	Invasive only (n=114)
Histological type	ductal	39 (97.5%)	95 (84.1%)
	lobular	0 (0.0%)	8 (7.1%)
	other	1 (2.5%)	10 (8.8%)
SBR grading	I	7 (17.9%)	23 (20.4%)
	II	22 (56.4%)	60 (53.1%)
	III	10 (25.6%)	30 (26.5%)
Differentiation	1	1 (2.6%)	7 (6.2%)
	2	25 (64.1%)	51 (45.1%)
	3	13 (33.3%)	55 (48.7%)
Anisonucleosis	1	2 (5.1%)	9 (8.0%)
	2	26 (66.7%)	78 (69.0%)
	3	11 (28.2%)	26 (23.0%)
Mitoses	1	11 (28.2%)	31 (27.4%)
	2	9 (43.1%)	41 (36.3%)
	3	19 (48.7%)	41 (36.3%)
Emboli	absent	33 (82.5%)	97 (85.1%)
	ambiguous	1 (2.5%)	5 (4.4%)
	present	6 (15.0%)	12 (10.5%)
Necrosis (% of tumour area)	mean±SD	1.6±4.1	3.1±9.9
Inflammation	scarce	24 (61.5%)	86 (76.1%)
	moderate	12 (30.8%)	19 (16.8%)
	important	3 (7.7%)	8 (7.1%)
Type of inflammatory cells	lymphocytes	38 (97.4%)	107 (94.7%)
	plasmacytes	1 (2.6%)	5 (4.4%)
	mixed	0 (0.0%)	1 (0.9%)
Abundance of stroma	mild	6 (15.4%)	19 (16.8%)
	moderate	24 (61.5%)	59 (52.2%)
	abundant	9 (23.1%)	35 (31.0%)
Type of stroma	oedema	9 (23.1%)	22 (19.5%)
	hyalin	14 (35.9%)	58 (51.3%)
	fibroelastosic	16 (41.0%)	31 (27.4%)
	vascular	0 (0.0%)	2 (1.8%)

lower ($p<0.0001$, Student's *t*-test) with fewer menopaused patients (48.9%) and the mean pT (22.6±11.2 mm) was also smaller ($p=0.0001$, Student's *t*-test).

Before surgery, mean±SD HER-2 ECD was 12.48±7.08 ng/ml (range 6.30-82.00 ng/ml, median 11.15 ng/ml) and 21/157 (13.4%) sera had an HER-2 ECD concentration ≥15 ng/ml. The distribution of HER-2 ECD concentrations was found to be non gaussian.

Histology. Among the 157 tumours, 40 (25.48%) showed both invasive and intraductal components, 3 (1.91%) were pure *in situ* carcinomas and 114 (72.61%) were pure invasive tumours. A detailed description of the histopathological characteristics of the tumours is given in Tables II and III. For 16 cases the intraductal component was of comedo-type; there was no Paget's disease in the series. No significant relationships were found between the different criteria listed in Tables II and III, with two exceptions. Anisonucleosis was found to be related to nuclear grade ($p<0.0007$, χ^2 test), with all SBR grade III anisonucleosis (n=11) corresponding to tumours with nuclear grade III in the intraductal component. Peri-ductal inflammation was also associated with nuclear grade ($p=0.0380$, χ^2 test).The presence of periductal inflammation was recorded in nuclear grade 3 tumours for 14/21 (66.67%) cases.

Immunohistochemistry. There was a general agreement between the two antibodies for scores and percent of stained cells (Table IV). The comparison among tumours with an invasive component (n=154) showed a contingency coefficient of 0.814 for Dako and CB 11 IHC scores ($p<0.0001$, χ^2 test) and Spearman rho was 0.84 for percent of stained cells ($p<0.00011$). For tumours with an intraductal component (n=43), the contingency coefficient was 0.739 for IHC scores ($p<0.0001$, χ^2 test) and Spearman rho was 0.914 for percent of stained cells ($p<0.0001$). With the Dako antibody, we never obtained a cytoplasmic staining in intraductal tumours. However, a gap was evidenced when considering the distribution of the positive scores obtained with each of the antibodies, with a majority of 2+ scores obtained with the CB 11 mAb (Table IV). With both antibodies, we recorded

Table III. *Histopathological data. Part II : intraductal component.*

Variables	Categories	Invasive and intraductal (n=40)	Intraductal only (n=3)
Histological type	comedocarcinoma	14 (35.0%)	2 (66.7%)
	cribriform	10 (25.0%)	0 (0.0%)
	solid	9 (22.5%)	0 (0.0%)
	papillary	1 (2.5%)	0 (0.0%)
	mixed	6 (15.0%)	1 (33.3%)
Nuclear grade	1	4 (10.0%)	0 (0.0%)
	2	17 (42.5%)	1 (33.3%)
	3	19 (47.5%)	2 (66.7%)
Comedonecrosis	absence	16 (40.0%)	0 (0.0%)
	localized	14 (35.0%)	1 (33.3%)
	spread	8 (20.0%)	1 (33.3%)
	massive	2 (5.0%)	1 (33.3%)
Differentiation	undifferentiated	15 (37.5%)	2 (66.7%)
	differentiated	25 (62.5%)	1 (33.3%)
Importance (% of tumour area)	≤ 10	22 (55.5%)	0 (0.0%)
	11-75	14 (35.0%)	0 (0.0%)
	> 75	4 (10.0%)	3 (100.0%)
Diffusion at distance	no	30 (75.5%)	2 (66.7%)
	yes	10 (25.0%)	1 (33.3%)
Periductal inflammatory infiltration	absence	26 (65.0%)	0 (0.0%)
	weak and focal	9 (22.5%)	1 (33.3%)
	moderate	4 (10.0%)	1 (33.3%)
	intense	1 (2.5%)	1 (33.3%)

significantly more positive cases in grade III tumours (14/40, 35.0% for Dako antibody 3+ tumours, $p=0.005$ and 16/40, 40.0% 2+ and 3+ tumours, for CB 11 mAb, $p=0.003$, χ^2 test).

In the mixed tumours (n=40), there was a good agreement between the staining scores of invasive and intraductal components (weighted kappa = 0.644 when using the Dako antibody and 0.628 for the CB 11 antibody).

In the intraductal component, staining scores and percent of labelled cells were related to intraductal histological types when using Dako antibody ($p=0.0475$, χ^2 test and 0.0027, Kruskal Wallis test, respectively). This relationship is attributable to the high proportion (11/16, 68.8%) of comedo-type tumours in the intraductal Dako 3+ tumours. For the CB 11 antibody, the same comparisons led to non significant results for scores but to a significant relationship when considering the proportion of stained cells according to intraductal histological type ($p=0.0007$, Kruskal Wallis test). Among the 16 comedo-type tumours, 3/3 were pure intraductal tumours and 13 mixed intraductal and invasive tumours. The 3 pure intraductal tumours were all 3+ when using Dako antibody and 2+ with CB 11 antibody, with a mean proportion of stained cells of 95.0±5.0% and 90.0±8.7%, respectively. The comparison of staining scores or proportions of stained cells between comedo-type tumours and the other tumours with each of the antibodies led to significant differences in all instances ($p<0.005$, χ^2 or Mann Whitney tests).

Relationships between pre-treatment HER-2 ECD concentration and histological characteristics of the paired tumours. Among all the clinico-biological variables recorded at diagnosis (Table I), pre-treatment HER-2 ECD concentrations were found to be positively correlated only to tumour clinical ($p=0.0022$) and pathological size ($p=0.0017$, Spearman rank test).

None of the characteristics of the invasive component, namely histological type or grade or its separate elements, presence of emboli, proportion of necrosis, inflammation, abundance or type of stroma were significantly linked to HER-2 ECD concentration taken as a continuous variable (Table II).The mean HER-2 ECD concentration was more elevated in mixed invasive and intraductal tumours (14.5±13.4 ng/ml) than in pure invasive tumours (11.8± 2.4 ng/ml), ($p=0.040$, Student's *t*-test). Dichotomised by the HER-2 ECD cut-off, cases with elevated HER-2 ECD (n=21) corresponded to larger tumour clinical and pathological sizes (mean T: 57.1±30.2 mm *versus* 31.2±14.4 mm, $p<0.0001$ and mean pT: 37.8±17.6 mm *versus* 24.9±10.5 mm, $p=0.0008$, Mann Whitney test) and higher SBR grades ($p=0.0465$, χ^2 test). Non significant differences in tumour histological types were found according to HER-2 ECD subgroups.

All the variables describing the intraductal component were found to be unrelated to the corresponding HER-2 ECD concentrations, with one exception (Table III). A higher proportion of HER-2 ECD over 15 ng/ml was observed in tumours showing a spread or massive comedonecrosis as compared to tumours without or with focal comedonecrosis (5/12 (41.7%) *versus* 2/31 (6.5%), $p=0.0123$, Fisher's exact test). The proportion of intraductal carcinoma and its histological type were found to be unrelated to HER-2 ECD levels. As expected, a higher proportion of HER-2 ECD over 15 ng/ml was found in comedocarcinomas when compared to other tumour types (5/16, 31.2% *versus* 2/27, 7.4%, respectively, $p=0.041$, χ^2 test).

Relationships between pre-treatment HER-2 ECD concentration, immunohistochemical scores and proportion of stained cells. The results are summarized in Table V. Low concentrations of HER-2 ECD, without any case over the cut-off, were constantly observed with each of the antibodies in tumours showing a cytoplasmic staining. Total concordance in the invasive component (cytoplasmic staining excluded), *i.e.* HER-2 ECD < 15 ng/ml for scores 0-2+ (n=111) and ≥15 ng/ml for 3+ cases (n=13) was 124/149 (83.22%) when using Dako antibody. For CB 11 antibody, total concordance was 116/144 (80.56%) if 2+ and 3+ cases were mixed.

Table IV. *Results of HER-2 immunohistochemistry.*

	Dako antibody						CB 11 antibody					
	scores					% stained cells	scores					% stained cells
	0	1	2	3	4	Mean±SD	0	1	2	3	4	Mean±SD
	Tumours with intraductal and invasive component (n=40)											
Intraductal component	11 (27.5%)	9 (22.5%)	9 (22.5%)	11 (27.5%)	0 (0.0%)	40.7±44.0	15 (37.5%)	8 (20.0%)	14 (35.0%)	2 (5.0%)	1 (2.5%)	30.8±40.9
Invasive component	15 (37.5%)	10 (25.0%)	3 (7.5%)	10 (25.0%)	2 (5.0%)	36.7±41.3	15 (37.5%)	9 (22.5%)	10 (25.0%)	2 (5.0%)	4 (10.0%)	33.8±39.9
	Tumours with intraductal component only (n=3)											
	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	95.0±5.0	0 (0.0%)	0 (0.0%)	3 (100.0%)	0 (0.0%)	0 (0.0%)	90.0±8.7
	Tumours with invasive component only (n=114)											
	69 (60.5%)	18 (15.8%)	4 (3.5%)	17 (14.9%)	6 (5.3%)	21.9±36.8	77 (67.5%)	10 (8.8%)	15 (13.2%)	3 (2.6%)	9 (7.9%)	19.7±35.6

0= no staining, 1+ = incomplete membrane staining, 2+ = complete but weak membrane staining, 3+ = complete and strong membrane staining, (any + score attributed if there is > 10% of stained cells), 4 = cytoplasmic staining.

For tumours scored 0 to 2+ (Dako antibody) or 0 and 1+ (CB 11 antibody), 8/119 (6.72%) and 8/111 (7.21%) of the cases, respectively, had an HER-2 ECD concentration over 15 ng/ml (Table V). All those tumours had an invasive component and 3 of them also showed an intraductal component. Detailed analysis of these 8 cases showed that 2 patients had borderline HER-2 ECD elevation (<16 ng/ml) before and after primary treatment and presented with severe liver dysfunction unrelated to cancer. For 3 other patients, the IHC was performed on small core-needle biopsies (14 G) before neoadjuvant treatment. Two of them had large tumours (pT: 45 and 50 mm); one patient developed a contralateral breast tumour, while the second one showed normalized HER-2 ECD values after primary treatment and did not relapse. For the last patient, the original 2 + Dako and CB 11 scores were lowered to 1+ after review. When the histological features of these 8 tumours were compared to the whole series, the sole difference was the mean percent of positive cells, which was 8.8±13.6% (Dako staining) and 4.4±10.5% (CB11 staining).

In the invasive component, after discarding tumours showing a cytoplasmic staining, mean HER-2 ECD levels were significantly related to IHC scores (p=0.0166 for Dako antibody and p=0.0628 for CB 11 antibody, Kruskall-Wallis test, Figures 1 and 2). As expected, the highest mean HER-2 ECD concentration and the highest proportion of elevated cases (13/27 (48.1%) ≥15 ng/ml) were recorded in the Dako 3+ tumours (Table V). When using CB 11 antibody, the highest mean HER-2 ECD was observed in 2+ tumours with 10/25 cases with HER-2 ECD over 15 ng/ml. In the CB 11 3+ subgroup, the mean HER-2 ECD was 14.3±5.3 ng/ml and 3/5 cases were over 15 ng/ml. Spearman rank tests with the proportion of stained cells and HER-2 ECD concentration

Table V. *Relationships between HER-2 ECD concentration in pre-treatment sera and immunohistochemical scores.*

Scores*	Dako antibody			CB 11 antibody		
	HER-2 ECD (ng/ml)					
	n	mean±SD	>15 ng/ml	n	mean±SD	>15 ng/ml
Invasive component						
0	84	11.5±1.8	5 (6.0%)	92	11.4±1.8	6 (6.5%)
1+	28	11.4±2.1	3 (10.7%)	19	11.2±2.2	2 (10.5%)
2+	7	10.2±2.4	0 (0.0%)	25	17.6±16.4	10 (40.0%)
3+	27	17.8±15.8	13 (48.1%)	5	14.3±5.6	3 (60.0%)
4	8	11.1±1.0	0 (0.0%)	13	11.6±1.3	0 (0.0%)
p value**		0.0337			NS	
Intraductal component						
0	11	12.2±2.8	2 (18.2%)	15	12.0±2.4	2 (13.3%)
1+	9	12.1±2.3	1 (11.1%)	8	11.5±2.7	1 (12.5%)
2+	9	10.8±1.1	0 (0.0%)	17	17.7±20.1	3 (17.6%)
3+	14	19.7±21.9	4 (28.6%)	2	14.7±7.6	1 (50.0%)
4	0	-	-	1	13.5	0 (0.0%)
p value**			NS		NS	

0= no staining, 1+ = incomplete membrane staining, 2+ = complete but weak membrane staining, 3+ = complete and strong membrane staining, (any + score attributed if there is > 10% of stained cells), 4 = cytoplasmic staining.
** Kruskal Wallis test. NS= non significant difference.

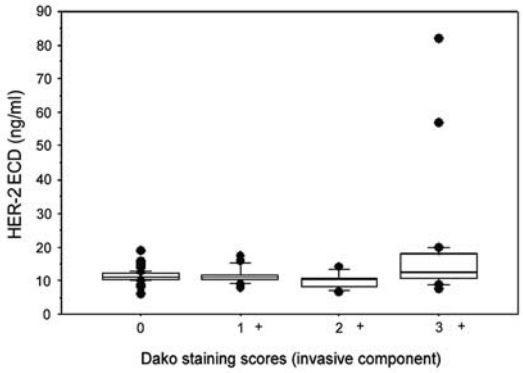

Figure 1. *Box plot of HER-2 ECD concentration according to Dako scores for the invasive component (n=149).*

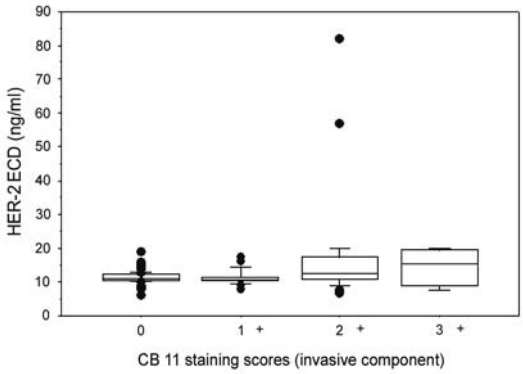

Figure 2. *Box plot of HER-2 ECD concentration according to CB 11 scores for the invasive component (n=144).*

resulted in a correlation of borderline significance for Dako antibody (p corrected for ties 0.0614) and a positive correlation when using the CB 11 antibody ($p=0.0455$).

In the intraductal component, none of the comparisons led to significant differences in mean HER-2 ECD concentration or proportion of stained cells with both antibodies (Table V).

Histological characteristics contributing to pre-treatment HER-2 ECD concentration. We performed a stepwise logistic regression analysis to identify the significant covariates contributing to normal or elevated (≥15 ng/ml) baseline HER-2 ECD concentrations in the group of pure invasive carcinomas (cytoplasmic staining excluded). The candidate variables included: tumour pathological size, as a continuous variable or dichotomised (≤20 mm or >20 mm), number of invaded lymph nodes or dichotomised into negative/positive pN, or divided into 3 groups (negative,1- 3, > 3 pN), Dako score 3+ *versus* other scores, CB 11 score 3+ *versus* other scores and the importance of inflammation in the invasive component. The significant parameters explaining pre-treatment HER-2 ECD concentration were the existence of a Dako 3+ score and tumour pathological size. The Hosmer-Lemeshow test indicated a goodness of fit (Chi square =10.0893, $p=0.1836$) (Table VI).

The Mann-Whitney test in the Dako 3+ subgroup, with HER-2 ECD dichotomised according to the cut-off and pT as a continuous variable, showed that mean±SD pT was 24.9±6.3 mm when HER-2 ECD was <15 ng/ml and 42.7±18.9 mm when HER-2 ECD was ≥15 ng/ml ($p=0.0062$, Mann-Whitney test). The same analysis performed with the CB 11 2+ and 3+ subgroup led to similar results (mean±SD pT 24.4±7.1 mm for HER-2 ECD under cut-off and 42.7±18.9 mm for the opposite group, $p=0.0036$, Mann-Whitney test). From the bivariate scattergram, the threshold to obtain a HER-2 ECD concentration over 15 ng/ml is 28-30 mm for both antibodies (Figures 3 and 4).

Table VI. *Results of logistic regression analysis.*

Regression parameters	Regression coefficients	Standard error	95% Wald confidence interval	*p* value
Intercept	- 4.6413	0.8934	[-6.3924 ;-2.8902]	< 0.0001
Dako score 3+ (invasive component)	2.0511	0.5925	[0.8898 ; 3.2123]	0.0005
pT (mm)	0.0713	0.0246	[0.0231 ; 0.1196]	0.0038

Establishment of a cut-off for serum HER-2 ECD on the basis of immunohistochemical scores. Although 15 ng/ml is the most frequently used cut-off for HER-2 ECD elevation, there is still no general consensus about a discriminant threshold for clinical use. We, therefore, performed an ROC analysis based on the assumption that Dako 3+ or CB 11 2+ and 3+ tumours were the positive cases and all other scores the negative cases. For Dako staining, the best fit for sensitivity (48.1%) and specificity (93.7%) was at 14.45 ng/ml, with an area under the ROC curve of 0.672 (95% CI (confidence interval) 0.592-0.745). With CB 11 staining, we obtained a lower cut-off (13.60 ng/ml) with a sensitivity of 48.3% and a specificity of 92.0% (area under the ROC curve 0.586, 95% CI 0.590-0.743). We thus set our cut-off at 15 ng/ml for simplification.

Discussion

IHC analysis of primary breast cancers stained by Dako and CB 11 antibodies, the most widely used antibodies, showed an overall agreement between each other. Dako and CB 11 antibodies recognize an intracytoplasmic domain of the HER-2 receptor, CB 11 recognizes an epitope near the C-terminus of the internal domain of the HER-2 receptor (19).

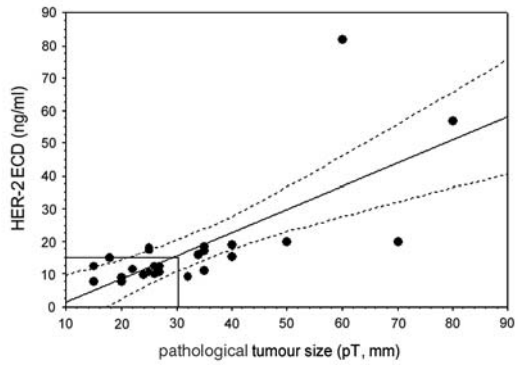

Figure 3. *Bivariate scattergram of HER-2 ECD concentration in the Dako 3+ group according to the pathological size of the tumours (n=26).*

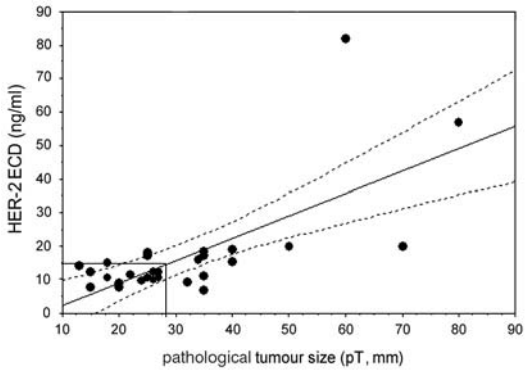

Figure 4. *Bivariate scattergram of HER-2 ECD concentration in the CB 11 2+ and 3+ group according to the pathological size of the tumours (n=29).*

However, by using the same Dako scoring system, our series evidenced that a large number of score 2+ tumours with CB 11 antibody corresponded to tumours scored 3+ with Dako antibody. Since the technical procedures were the same, this shift might be primarily due to the influence of the strong staining given by the Dako antibody as compared to CB 11 mAb staining, which was, in our work, evaluated after the Dako staining by the pathologist. Differences in antibody affinity and specificity might also contribute to that shift. The latter hypothesis cannot be assessed since the exact characteristics of the Dako antibody are not published. In the literature, there is a current discussion about IHC 2+ tumours, considered by some positive (2+ and 3+), while others consider 2+ as an undetermined result requiring additional analysis (FISH test). For clinical use, the difficulty of differentiating 2+ from 3+ scores may not be crucial, since a recent study on a large cohort of patients concluded that 2+ tumours should be considered as positive cases (5).

We found a correlation between tumour IHC scores obtained by currently used methods and corresponding HER-2 ECD levels measured by the Bayer automated serum assay, although the targeted molecular domains and protein function are different. A similar observation was reported by Molina *et al.* (20). IHC methods recognize intracytoplasmic domains of the entire HER-2 receptor. The serum HER-2 assay measures the extracellular domain of the HER-2 receptor by using two monoclonal antibodies recognizing two independent epitopes (6). The exact mechanisms of HER-2 ECD shedding and its regulation are not yet completely known, but involve basement membrane degradation by matrix metalloproteinases.

Analysis of the discrepancies between IHC and serum assays, *i.e.* HER-2 ECD > 15 ng/ml and scores 0 or 1+ by Dako or CB 11 staining, highlighted several different problems. First, the causes of so called "non specific elevations" of HER-2 ECD are not yet well known, however, abnormal HER-2 ECD levels have been reported in patients with liver dysfunction (21). Tissue heterogeneity leading to sampling problems, especially when analysing small biopsies, is also likely to be encountered when performing IHC. The last observation concerns the Dako scoring system, primarily designed as a predictive test for response to trastuzumab and not for the precise evaluation of tumour HER-2 overexpression. This system involves regarding negative tumours as those showing less than 10% of stained cells and, from the present results, this assumption may not be appropriate in the case of large tumours.

We also sought to define, among the different characteristics of tumour invasive and intraductal components, those contributing to measurable circulating levels of HER-2 ECD. Apart from the well known link with comedo-type carcinomas, none of the recorded histological characteristics showed significant relationships with HER-2 ECD, including the importance of tumour necrosis (22). As previously reported, we found a concordance between HER-2 overexpression in the intraductal and invasive components of mixed tumours and a frequent HER-2 overexpression in grade III tumours (23). We also found a more frequent HER-2 overexpression in high-grade intraductal tumours (24). However, our results showed that the intraductal component, surprisingly, does not contribute to a large extent to the HER-2 ECD circulating level, and this is illustrated by the fact that all the pure intraductal Dako 3+ tumours in our series had an HER-2 ECD concentration under 15 ng/ml. Clearly, little is known about the fate of intraductal biological markers and the mechanisms by which they gain access to the circulation.

The only features found to be linked with HER-2 ECD circulating levels were IHC scores for the invasive component and tumour size. We cannot exclude that the degree of nodal involvement, found to be unrelated to HER-2 ECD levels in the present subgroup but significantly related to nodal status in the whole series of 701 patients, could also contribute to HER-2 ECD levels. The gap between the reported frequencies for tissue HER-2 overexpression (20-30%) and

for elevated pre-treatment HER-2 ECD in stages I-III breast cancers (<10%) might be explained by the existence of a threshold of invasive tumour size necessary to observe elevated circulating HER-2 ECD. Besides, existence of circulating micrometastases overexpressing HER-2 might also contribute to elevated levels of HER-2 ECD (25).

To conclude, elevated concentrations of HER-2 ECD at diagnosis are linked to tumour HER-2 overexpression in the invasive component and are found when tumours exceed 28-30 mm. This information may be useful when the primary tumour is not available for immunohistochemical analysis. Furthermore, as a highly reproducible method, HER-2 ECD assays could improve the performance of tissue testing and represent an alternative for initiation of trastuzumab therapy in the metastatic setting (26, 27).

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References

1 Ross JS and Fletcher JA: HER-2/*neu* (c-erbB2) gene and protein in breast cancer. Am J Clin Pathol *112*: S53-67, 1999.

2 Coussens L, Yang-Feng TL, Liao Y-C *et al*: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science *230*: 1132-9, 1985.

3 Yeh IT: Measuring HER-2 in breast cancer. Am J Clin Pathol *117* (suppl): S26-S35, 2002.

4 Roche PC, Suman VJ, Jenkins RB *et al*: Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. J Natl Cancer Inst *94*: 855-85, 2002.

5 Owens MA, Horton DC and Da Silva M: HER2 amplification ratio by fluorescence *in situ* hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. Clin Breast Cancer *5*: 63-69, 2004.

6 Carney WO, Hamer PJ, Petit D *et al*: Detection and quantitation of the human *neu* oncoprotein. J Tumour Mark Oncol *6*: 53-72, 1991.

7 Carney WP, Neumann R, Lipton A *et al*: Monitoring the circulating levels of the HER2/*neu* oncoprotein in breast cancer. Clin Breast Cancer *5*: 105-116, 2004.

8 Saghatchian M, Guepratte S, Hacene K *et al*: Serum HER-2 extracellular domain: relationship with clinicobiological presentation and prognostic value before and after primary treatment in 701 breast cancer patients. Int J Biol Markers *19*: 14-22, 2004.

9 Pichon MF, Hacene K, Guepratte S *et al*: Serum HER-2 extracellular domain (ECD) before the first metastasis in 128 breast cancer patients. Clin Lab *50*: 163-170, 2004.

10 Payne RC, Allard JW, Anderson-Mauser L *et al*: Automated assay for HER-2/*neu* in serum. Clin Chem *46*: 175-182, 2000.

11 Bloom HJG and Richardson WW: Histological grading and prognosis in breast cancer. Br J Cancer *11*: 339-377, 1957.

12 Holland R, Peterse JL, Millis RR *et al*: Ductal carcinoma *in situ*: a proposal for a new classification. Semin Diagnostic Pathol *11*: 167-180, 1994.

13 Schmitt SJ, Conolly, JL Harris JR *et al*: Pathologic predictors of early local recurrence in Stage I and II breast cancer treated by primary radiation therapy. Cancer *53*: 1049-57, 1984.

14 Penault-Llorca F, Balaton A, Sabourin JC *et al*: Evaluation immunohistochimique du statut HER2 dans les carcinomes infiltrants: mise au point du protocole technique et de la lecture des résultats-recommandations. Ann Pathol *22*: 150-157, 2002.

15 Ellis IO, Dowsett M, Bartlett J *et al*: Recommendations for HER2 testing in the UK. J Clin Pathol *53*: 890-892, 2000.

16 Graziano C: HER-2 breast assay, linked to Herceptin, wins FDA okay. CAP Today *12*: 13-16, 1998.

17 Jacobs TW, Gown AM, Yazidji H *et al*: Specificity of the HercepTest in determining HER-2/*neu* status of breast cancers using the United States Food and Drug Administration-approved scoring system. J Clin Oncol *17*: 1983-1987, 1999.

18 Press MF, Slamon DJ, Flom KJ *et al*: Evaluation of HER-2/*neu* gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimen. J Clin Oncol *20*: 3095-3105, 2002.

19 Corbett PI, Henry JA, Angus B *et al*: NCL-CB 11 a new monoclonal antibody recognizing the internal domain of the c-erbB-2 oncogen protein effective for use on formalin-fixed, paraffin- embedded tissue. J Pathol *161*: 15-25, 1990.

20 Molina R, Fillela X, Zanon G *et al*: Prospective evaluation of tumor markers (c-erbB-2 oncoprotein CEA and CA 15.3) in patients with locoregional breast cancer. Anticancer Res *23*: 1043-1050, 2003.

21 Imoto S, Kitoh T and Hasebe T: Serum c-erbB-2 levels in monitoring of operable breast cancer patients. Jpn J Clin Oncol *29*: 336-339, 1999.

22 Van De Vijver MJ, Peterse JL, Wolter J *et al*: Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma *in situ* and limited prognostic value in stage II breast cancer. N Engl J Med *319*: 1239-45, 1988.

23 Warnberg F, Nordgren H, Bergkvist L *et al*: Tumour markers in breast carcinoma correlate with grade rather than with invasiveness. Br J Cancer *85*: 869-874, 2001.

24 Craig Allred D, Clark GM, Molina R *et al*: Overexpression of HER-2/*neu* and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancer. Hum Pathol *23*: 974-979, 1992.

25 Braun S, Hepp F, Sommer HL *et al*: Tumour-antigen heterogeneity of disseminated breast cancer cells: implications for immunotherapy of minimal residual disease. Int J Cancer (Pred Oncol) *84*: 1-5, 1999.

26 Köstler W, Schwab B, Singer CF *et al*: Monitoring of serum Her-2/*neu* predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer. Clin Cancer Res *10*: 1618-1624, 2004.

27 Bethune-Volters A, Labroquere M, Guepratte S *et al*: Longitudinal changes in serum HER-2/*neu* oncoprotein levels in trastuzumab-treated metastatic breast cancer patients. Anticancer Res *24*: 1083-1090, 2004.

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