Pergamon

European Journal of Cancer, Vol. 34, No. 4, pp. 476–481, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(97)10069-7

Original Paper

Quantitative Determination of c-erbB-2 in Human Breast Tumours: Potential Prognostic Significance of Low Values

S. Koscielny, Ph. Terrier, A. Daver, J. Wafflart, J. Goussard, G. Ricolleau, C. Delvincourt and J.C. Delarue

Groupe de Biopathologie Tissulaire et Moléculaire, Fédération des Centres de Lutte contre le Cancer, 101 rue de Tolbiac, 75654 Paris, Cedex 13, France

The purpose of this prospective multicentric study was to quantify the c-erbB-2 protein and investigate its relationship with DNA amplification and with various prognostic parameters of breast cancer. A total of 1062 primary operable human breast tumours were collected from six French anticancer centres. The c-erbB-2 protein was measured using an enzymoimmunoassay using two monoclonal antibodies directed against the extracellular domain of the protein. The results were expressed in arbitrary units/mg membrane protein (AU) after adjustment for the anticancer centre. A significant association was found between the dosage of the protein and DNA amplification ($P = 0.0001$). A value of 200 AU was found to maximise sensibility and specificity and was chosen as a cut-off for overexpression. Significant associations were found between c-erbB-2 values and oestrogen receptor (ER) ($P = 0.01$), progesterone receptor (PgR) ($P = 0.0001$) and histological grading ($P = 0.01$). The extreme high values (above the mean plus one standard deviation, S.D.) were significantly more numerous in ER^- ($P = 10^{-16}$), PgR^- ($P = 10^{-14}$) and grade III ($P = 10^{-8}$) tumours. The extreme low values (below the mean minus one S.D.) were significantly more numerous in ER^- ($P = 10^{-9}$) and PgR^- ($P = 0.02$) tumours. This prospective study confirms that high c-erbB-2 protein values are linked to poor prognostic factors and shows for the first time that low values are also linked to hormone receptor negative tumours, suggesting that these low values might also have a negative prognostic significance. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: breast tumours, c-erbB-2, prognostic factors
Eur J Cancer, Vol. 34, No. 4, pp. 476–481, 1998

INTRODUCTION

THE *c-erbB-2* gene (*HER2/neu*) is a member of the class of oncogenes associated with tyrosine protein kinase [1, 2]. The protein encoded by *c-erbB-2* is a 185 kDa transmembrane receptor (p185) with intracellular, transmembrane and extracellular domains [3, 4]. The *c-erbB-2* gene has been reported amplified in 25–30% of human breast and ovarian cancers [5, 6]. Slamon and associates [7] demonstrated that *c-erbB-2* amplification is an independent predictor of both relapse and overall survival.

DNA amplification of the *c-erbB-2* gene is the most common mechanism of activation of the gene, leading to overexpression of the c-erbB-2 protein. Another mechanism of *c-erbB-2* activation is overexpression of the c-erbB-2 protein without gene amplification [7]. Measurement of c-erbB-2 protein overexpression is, therefore, thought to be a better indicator of *c-erbB-2* activation than DNA amplification [8].

The most accurate methods for detecting the c-erbB-2 protein are the Western blot technique and immunoprecipitation [9]. However, these methods are not suitable for routine laboratory use. Immunohistochemical staining using paraffin-embedded material is the technique most commonly employed to determine protein expression. Immunohistochemical staining positivity ranges from 10–40% in primary breast cancer (see [10], Table 6 for review). Although the technique is reliable, the criteria used to assess the results of staining are inconsistent and variations in tissue fixation have produced conflicting results [11]. All these problems can be avoided by extracting p185 from the cell membrane with a

Correspondence to J.C. Delarue. Institute G. Roussy, 94805 Villejuif, France.
Received 23 Jun. 1997; revised 15 Sep. 1997; accepted 9 Oct. 1997.

detergent and quantifying it with a recently developed enzyme immunoassay (EIA) (Triton Diagnostics, Alameda, California, U.S.A.).

This report describes the use of this EIA to quantify the c-erbB-2 protein in extracts of 1062 primary breast cancer tissues collected from six French anticancer centres. The results obtained were then compared with those acquired with DNA amplification. The c-erbB-2 protein was quantitatively assessed and the results compared with other prognostic parameters of primary breast cancer. In this prospective study, the follow-up of patients is too small to appreciate directly the prognostic significance of the c-erbB-2 protein.

MATERIAL AND METHODS

Patients

Tumour tissues were collected from 1062 patients treated between September 1993 and October 1994 for a primary breast cancer in six French anticancer centres (Angers, 336; Bordeaux, 222; Caen, 161; Nantes, 150; Villejuif, 117; Reims, 76). All patients underwent either radical mastectomy or tumorectomy and had received no chemo-, endocrino- or radiotherapy prior to surgery. The population is described in Table 1.

Table 1. *Patients' clinicobiological characteristics*

Characteristics	Total	(%)
Overall population	1062	(100)
Age		
≤ 50 years	354	(33)
51–60 years	234	(22)
> 60 years	474	(45)
T*		
0 or 1	376	(35)
2 or 3 or 4	522	(49)
ND	164	(15)
Nodal involvement		
Negative	700	(66)
Positive	281	(26)
ND	81	(8)
Histological grading†		
I	177	(17)
II	443	(42)
III	282	(27)
ND	160	(15)
Histological types		
Ductal	855	(81)
Lobular	113	(11)
Others	65	(6)
ND	29	(3)
ER‡		
Negative	251	(24)
Positive	806	(76)
ND	5	(< 1)
PgR‡		
Negative	413	(39)
Positive	647	(61)
ND	2	(< 1)

*According to the TNM classification. †According to SBR [12]. ‡Positive if >10 or 15 fmol/mg cytosol protein, depending on the anticancer centre. ND, not determined; ER, oestrogen receptors; PgR, progesterone receptors.

Oestrogen receptor (ER) and progesterone receptor (PgR) determination

Oestrogen receptors (ERs) and progesterone receptors (PgRs) were determined on samples stored in liquid nitrogen. Two types of ER and PgR assays were performed: a radioligand binding assay using dextran-coated charcoal [13] in three centres: Angers, Bordeaux, Caen; and an enzyme immunoassay using a kit obtained from Abbott Laboratories (North Chicago, Illinois, U.S.A.) in the three other centres: Nantes, Villejuif, Reims. Good correlation has previously been established between these two methods [14, 15] and the cut-off level for positivity was 10 or 15 fmol/mg cytosol protein according to the different centres. All participating centres performed continuous quality control studies in collaboration with other European laboratories in the EORTC receptor group.

EIA for c-erbB-2 protein

After receptor determination, pelleted tumour tissue was resuspended in extraction solution containing 1% Triton X-100 provided in the kit. The c-erbB-2 protein in the tissue extract was assayed using an EIA kit, according to the manufacturer's instructions (Triton Diagnostics, Alameda, California, U.S.A.). The monoclonal antibodies (MAbs) in this assay (Tab 259 and Tab 257) are reactive with the extracellular domain of the c-erbB-2 protein [16]. The sensibility and specificity of the kit have been previously reported [17].

Pellet protein content was determined using the Pierce method (bicinchoninic acid) which is compatible with the presence of detergent and results were expressed in arbitrary units per mg of membrane protein.

DNA amplification

Total DNA was isolated with a kit using a phenol–chloroform extraction followed by isopropanol precipitation (Applied Biosystem). The concentration of the extract was adjusted to 0.5 mg/ml. Total DNA was analysed using the Southern blot method after enzymatic digestion by Hind III (80 U/10 μg DNA) with positive (SKBr-3 cells) and negative (lymphocytes and MCF-7 cells) controls. The 2700 bp c-erbB-2 probe (oncogene sciences) was used after ^{32}P-labelling by the Multiprime kit (Amersham, Buckinghamshire, U.K.). Hybridisation was at 65°C for 20 h. DNA concentrations were determined using quantitative densitometric scanning of appropriately exposed autoradiograms (Image Master, Pharmacia Biotech) and the ratio between the intensity of c-erbB-2 samples and the intensity of the c-erbB-2 negative control (lymphocyte) was calculated. *c-erbB-2* amplification was considered positive with a ratio ≥ 2.

Statistical analysis

The overall distribution of c-erbB-2 values was lognormal. In order to work with normally distributed values and, therefore, to respect the conditions of validity of the tests, computations were based on c-erbB-2 logarithms. Values equal to 0 were set at 1 before transformation. The geometric mean is defined as the exponential of the mean value computed from the logarithms and the standard deviation (S.D.) refers to logarithm values.

Association with DNA amplification and determination of a cut-off threshold for overexpression. The association between c-erbB-2 values and DNA amplification was tested using a Spearman correlation analysis. In order to determine a cut-off value indicative of overexpression, sensibility and specificity

curves were plotted. Overexpression was defined as a ratio ≥ 2 for the reference method (DNA amplification). Sensibility and specificity were calculated assuming cut-off values for the tested method (EIA) moving from 1 to 1000 AU. Sensibility was defined as true positive cases divided by true positive + false negative cases and specificity as true negative cases divided by true negative + false positive cases. True positive and true negative cases were those for which both methods were positive or negative, respectively. False positive and false negative cases were those for which only the method tested (EIA) was positive or negative, respectively.

Relationship between the c-erbB-2 level and prognostic parameters. The patients were classified according to their age and tumour parameters (clinical size, node involvement, histological grading, ERs and PgRs). The association between each of these parameters and c-erbB-2 level was assessed using the SAS®GLM procedure [18]. A two-way factorial model with interaction was specified to test the effects of the centre, the prognostic parameter and the interaction between the centre and the prognostic parameter. Significance tests were based on the type III sum of squares. This method takes into account the centre effect and allows the estimation of each prognostic parameter independently of the centre. The non-parametric methods currently used do not allow the heterogenity between the centres to be taken into account. Finally, adjusted mean c-erbB-2 values were computed for each prognostic factor category, assuming an equal number of patients in each category defined by the prognostic factor and the centre (LSMEAN).

Relationship between c-erbB-2 extreme values and prognostic parameters. Limits were defined as a function of the geometric mean and S.D. of the c-erbB-2 values in each centre. Low values are smaller than the geometric mean minus 1 S.D. and high values larger than the geometric mean plus 1 S.D. For each clinicobiological parameter, the proportions of patients with high or low values were compared in the different categories using a Fisher's exact test.

RESULTS

Intratumour c-erbB-2 distribution

Intratumour distribution of c-erbB-2 is shown in Table 2. A significant difference ($P < 0.001$) was observed between the geometric means of the six anticancer centres.

Table 2. Distribution of c-erbB-2 values according to the different anticancer centres

Characteristics	Number of cases	Geometric* mean c-erbB-2 value (AU)	Limit values (AU)	*P* value†
Overall population	1062	151	1–32 300	
Centre				
Angers	336	147	2–2100	< 0.001
Bordeaux	222	276	34–12 200	
Caen	161	153	1–8500	
Nantes	150	34	1–1146	
Villejuif	117	290	43–32 300	
Reims	76	211	20–7316	

*Geometric mean as defined in Material and Methods. †*P* value corresponding to the type III sum of squares in the GLM model.

Association with DNA amplification. Overexpression cut-off level

A significant association was observed between DNA amplification and c-erbB-2 overexpression measured using the EIA (Spearman $r = 0.31$, $n = 374$, $P = 0.0001$).

Sensibility and specificity curves plotted with the criteria defined in Material and Methods are shown in Figure 1. The cut-off for overexpressed cases is defined as the intersection point of both curves where sensibility and specificity are the highest. This cut-off corresponds to a value of 200 AU. In these conditions, 34% (361/1062) of cases were considered to overexpress c-erbB-2.

Correlation with clinicobiological parameters

As shown in Table 3, significant associations were noted between c-erbB-2 values and histological grading (grade I: 118 AU, grade II: 145 AU, grade III: 182 AU, $P = 0.01$), ER status (ER^-: 186, ER^+: 141, $P = 0.01$) and PgR status (PgR^-: 198, PgR^+: 130, $P = 0.0001$).

The percentages of high and low c-erbB-2 values were estimated in the different groups of patients for each clinicobiological parameter. A significantly higher percentage of high values was found in grade III as opposed to grade II and grade I ($P = 10^{-8}$) and in PgR^- tumours as opposed to PgR^+ tumours ($P = 10^{-14}$) (Table 4). These findings are consistent with the correlation previously shown in Table 3. However, we also found a significantly higher percentage of high values in ER^- tumours as opposed to ER^+ tumours ($P = 10^{-16}$), contrasting with the weak correlation shown in Table 3. We also observed a significantly higher percentage of low values in ER^- tumours as opposed to ER^+ tumours ($P = 10^{-9}$), and in PgR^- tumours, as opposed to PgR^+ tumours ($P = 0.02$).

DISCUSSION

Since the first report by Slamon and associates [7] on the prognostic significance of *c-erbB-2* oncogene amplification in patients with primary breast cancer, many studies have attempted to investigate whether c-erbB-2 overexpression is indeed a prognostic factor in breast cancer patients. Although its prognostic significance has been demonstrated in certain categories of patients, no conclusion has been reached for node-negative patients, the subgroup urgently in need of a

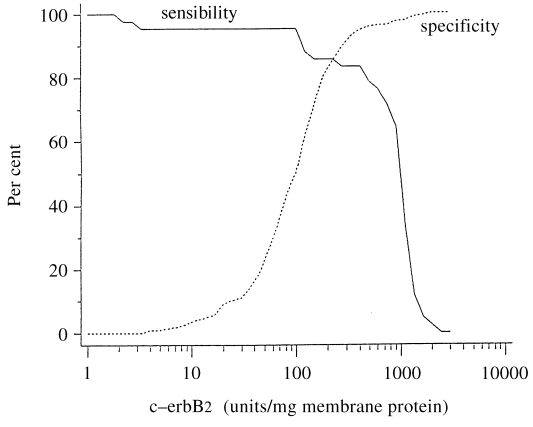

Figure 1. Sensibility (—) and specificity (....) curves as a function of the cut-off value (in units/mg membrane protein) chosen to express c-erbB-2 overexpression using the 'Triton Diagnostic' kit. The reference method used to appreciate the overexpressed (= positive) cases was DNA-amplification.

Table 3. Relationship between clinicobiological characteristics and c-erbB-2 mean values

Characteristics	Number of cases	Geometric mean* c-erbB-2 value	Adjusted geometric mean† c-erbB-2 value	*P* value‡
Overall population	1062	151		
Age				
≤ 50 years	354	161	159	NS
51–60 years	234	142	156	
> 60 years	474	148	144	
T§				
0 or 1	376	141	145	NS
2 or 3 or 4	522	165	164	
Nodal involvement				
Negative	700	156	154	NS
Positive	281	136	158	
Histological grading (SBR)				
I	177	133	118	0.01
II	443	138	145	
III	282	178	182	
Histology				
Ductal	855	155	156	NS
Lobular	113	113	130	
Other	65	197	201	
ER				
Negative	251	171	186	0.01
Positive	806	145	141	
PgR				
Negative	413	192	198	< 0.0001
Positive	647	129	130	

*Geometric mean as defined in Material and Methods. †Value recalculated assuming the same number of patients in all the centres. ‡*P* value corresponding to the type III sum of squares in the GLM model. §According to the TNM classification. ER, oestrogen receptors; PgR, progesterone receptors.

better prognostic index [19–24]. It has even been contended that *c-erbB-2* oncogene measurement is unjustified in routine clinical practice as it has yet to provide additional prognostic information to that offered by more traditional tests [25]. In our opinion, all these conflicting results and comments stem partly from the diversity of methods employed to determine c-erbB-2 and from the fact that immunohistochemistry, one of the most commonly used methods, has certain disadvantages: interpretation is subjective, antigenic immunoreactivity can be lost during fixation and it is difficult to quantify expression levels accurately.

This report presents the inherent advantages and pitfalls of a multicentric study. The large number of patients included in the study is the main advantage which is counterbalanced by variations in data from one centre to another, as presented in Table 2. These variations were not due to systematic differences between centres in the way the assay was carried out. Indeed, as part of the quality control process, identical specimens were analysed in each centre and the results did not show any significant difference from one centre to another (data not shown). Such differences could be ascribed to regional variation in the selection of patients due to differences in the behaviour of medical teams concerning the early diagnosis and management of breast cancer. However, the statistical analysis (see Material and Methods) took this centre effect into account and all the results were adjusted for it.

Until now, only amplification and/or overexpression of the *c-erbB-2* gene has been tested as a prognostic factor. In order to determine a cut-off for overexpression, the results obtained with the EIA described here were compared with those obtained with DNA amplification. The sensibility and specificity curves were plotted according to the previously defined criteria (see Material and Methods) with a single cut-off between negative and positive (= overexpressed) cases for the reference method and a variable one for the method investigated (Figure 1). The best cut-off point allowing discrimination of overexpressed cases obtained using the EIA was the point at which sensibility and specificity were the highest, i.e. the point at which the two curves intersected. This intersection point corresponded to 200 AU. With this cut-off threshold, 34% of the 1062 cases were considered to overexpress c-erbB-2. This percentage is consistent with previously published data [10, 26].

The relationship between c-erbB-2 expression and/or amplification and other prognostic factors has been extensively reported, exclusively in terms of positivity or negativity. In this study, the quantitative estimation of c-erbB-2 expression allowed better analysis of these relationships.

Firstly, we found a significant correlation ($P = 0.01$) between c-erbB-2 and histological grading (Table 3), which is in agreement with previous reports [19, 24, 27–29]. Secondly, we observed a strong inverse association between c-erbB-2 and PgR ($P < 0.0001$) but only a small association with the ER ($P = 0.01$) (Table 3). A similar result has already been reported by Watanabe and colleagues [29]. In the literature, the association between c-erbB-2 expression and/or amplification and the status of steroid receptors is controversial [5, 6, 8, 9, 21, 24, 26, 30–34]. The present study involved more than 1000 patients and we expected to find more important differences in c-erbB-2 values, according to hormonal status.

In an attempt to explain the weakness of these differences, we searched for associations between each clinicobiological parameter and low and high c-erbB-2 values, as presented in Table 4. The correlation between these extreme c-erbB-2 values and histological grading and PgR, respectively, is consistent with the correlation reported in Table 3 between the mean c-erbB-2 value and these parameters. Conversely, a significantly higher proportion of both extreme values was found in ER^- tumours. This finding has two consequences: (i) it explains why only a weak association was found between the ER status and the mean c-erbB-2 value; the results obtained with the distribution of low and high values, respectively, cancel out each other, (ii) low c-erbB-2 values are linked to poor prognostic factors (ER^- and, slightly, PgR^-) and are potentially of interest for prognosis. The same association between very low c-erbB-2 expression and ER negativity was also observed separately for each centre. This association was not always statistically significant due to the small number of cases in some centres. By way of a comparison, the same observation was made for the ER in recent papers which have shown that very high ER levels signal a poor prognosis, as dismal as that of ER^- tumours [35, 36]. Of course, this result must be confirmed by other studies, but the present report is the first to show that very low c-erbB-2 levels in breast tumours are linked to poor prognostic factors.

Table 4. High and low values of c-erbB-2 according to clinicobiological characteristics

Characteristics	Total number of patients	High values* Number of patients	(%)	*P*†	Low values‡ Number of patients	(%)	*P*†
Overall population	1062	133	(12.5)		110	(10.4)	
Age							
≤ 50 years	354	46	(13.0)	NS	26	(7.3)	0.04
51–60 years	234	27	(11.5)		24	(10.3)	
> 60 years	474	60	(12.7)		60	(12.7)	
T§							
0 or 1	376	36	(9.6)	0.02	37	(9.8)	NS
2 or 3 or 4	522	78	(14.9)		54	(10.3)	
Nodal involvement							
Negative	700	85	(12.1)	NS	75	(10.7)	NS
Positive	281	37	(13.2)		26	(9.3)	
Histological grading (SBR)							
I	177	6	(3.4)	10^{-8}	14	(7.9)	0.12
II	443	47	(10.6)		43	(9.7)	
III	282	60	(21.3)		38	(13.5)	
Histology							
Ductal	855	114	(13.3)	10^{-3}	89	(10.4)	NS
Lobular	113	4	(3.5)		11	(9.7)	
Other	65	13	(20.0)		8	(12.3)	
ER							
Negative	251	72	(28.7)	10^{-16}	54	(21.5)	10^{-9}
Positive	806	61	(7.6)		56	(6.9)	
PgR							
Negative	413	92	(22.3)	10^{-14}	54	(13.1)	0.02
Positive	647	41	(6.3)		56	(8.7)	

*High values: values larger than the geometric mean plus one S.D. †Chi square testing the equality of the proportion of high or low values among the different groups defined by the characteristic. ‡Low values: values smaller than the geometric mean minus one S.D. §According to the TNM classification. ER, oestrogen receptor; PgR, progesterone receptor.

Currently, we have no explanation for this observation. Although oncoprotein overexpression is widely considered as a poor prognostic factor, until now it has not been demonstrated that the absence of expression can also be equated with a poor prognosis. A plausible hypothesis is the concurrent regulation of several oncogenes during which the lack of expression of one gives rise to the overexpression of another. The inverse correlation recently observed by Robertson and associates [37] between c-erbB-2 and EGF receptors in human breast cancer is in keeping with this hypothesis. Another explanation could be that estradiol interferes with the metabolism of c-erbB-2 as previously demonstrated [38]. A loss of heterozygosity on chromosome 17q concerning the c-erbB-2 locus could also be considered.

Our study also found no correlation between the c-erbB-2 protein content and axillary lymph-node status and the literature is also discordant concerning this association. This result is in accordance with the hypothesis put forward by some investigators that these two factors are two different aspects of the pathogenesis of breast cancer dissemination: the nodal status reflects the relative chronological age whereas c-erbB-2 expression is an indicator of cellular kinetics and of tumour aggressiveness [39–41].

In conclusion, we cannot appreciate directly the prognostic significance of the c-erbB-2 oncoprotein on the basis of the present data because this is a prospective study and our patients underwent surgery too recently. The results do, however, confirm that high c-erbB-2 oncoprotein levels are linked to poor prognostic factors (histological grading III, ER^- and PgR^- tumours). We also show for the first time that very low c-erbB-2 oncoprotein levels are linked to ER^- and PgR^- tumours and perhaps carry a prognostic value. These results were achieved using a quantitative method to determine the c-erbB-2 oncoprotein whereas immunohistochemistry is only qualitative and only allows the detection of overexpressed cases. This EIA is a very simple, rapid procedure which could be standardised according to the usual quality control methodology used in laboratory tests. Alternatively, standardising tissue fixation methods and establishing minimum criteria for interpreting the results of c-erbB-2 protein staining are prerequisites if immunohistochemical staining is to provide valid and concordant results between institutes.

1. Weinberg RA. Oncogenes, antioncogenes and the molecular basis of multistep carcinogenesis. *Cancer Res* 1989, **49**, 3713–3721.
2. Van de Vijver M, Van de Bersselaar R, Deville P, Cornelisse C, Peterse J, Nusse R. Amplification of the neu (c-erbB-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked cerbA oncogene. *Mol Cell Biol* 1987, 7, 2019–2023.
3. Akyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c.erbB.2 gene: a 185 kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986, **232**, 1644–1646.

4. Schechter AL, Stern DF, Vaidyanathan L. The neu oncogene: an erb.B related gene encoding a 185,00-Mr tumour antigen. *Nature* 1984, **312**, 513–516.
5. Venter DJ, Tuzi NL, Kumar S, Gullick WJ. Overexpression of the protein in human breast carcinomas: immunohistological assessment correlates with gene amplification. *Lancet* 1987, **2**, 69–72.
6. 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.
7. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2 oncogene. *Science* 1987, **235**, 177–181.
8. Berger MS, Locher GW, Saurer S, *et al.* Correlation of c-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res* 1988, **48**, 1238-1243.
9. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2 neu oncogene protein and prognosis in breast cancer. *J Clin Oncol* 1989, 7, 1120–1128.
10. Terrier Ph, Mouriesse H, Loridon B, Gotteland M, May-Levin F, Delarue JC. Use of a polyclonal antibody for the determination of the prognostic value of c-erbB-2 protein overexpression in human breast cancer. *Acta Oncol* 1996, **35**, 23–30.
11. Singleton TP, Strickler JG. Clinical and pathologic significance of the c-erbB-2 (HER/neu) oncogene. *Pathol Annu* 1992, 165–190.
12. Bloom HJG, Richardson WW. Histological grading and prognosis in breast cancer: a study of 1409 cases of which 259 have been followed 15 years. *Br J Cancer* 1957, **11**, 359–377.
13. Martin PM, Bressot N, Delarue JC, *et al.* In Gest J, ed. *Evaluation des Moyens de Diagnostic du Cancer du Sein*. Paris, JMT Conseil, 1981, 173–185.
14. Delarue JC, Mouriesse H, Contesso G, *et al.* Récepteurs oestrogéniques dans les cancers du sein. Comparaison de 2 méthodes. *Ann Biol Clin* 1986, **44**, 629–633.
15. Blankenstein MA. Comparison of ligand binding assay and enzyme immunoassay of oestrogen receptor in human breast cancer cytosols. *Breast Cancer Res Treat* 1990, **17**, 91–98.
16. Langton BC, Creushaw MC, Chao LA, Stuart SG, Akita RW, Jackson JE. An antigen immunologically related to the external domain of gp185 is shed from nude mouse tumors overexpressing the c-erbB-2 (HER/neu) oncogene. *Cancer Res* 1991, **51**, 2593–2598.
17. Leitzel K, Teramoto Y, Sampson E, *et al.* Elevated soluble c-erbB-2 antigen levels in the serum and effusions of a proportion of breast cancer patients. *J Clin Oncol* 1992, **10**, 1436–1443.
18. SAS/STAT, User's guide, version 6, Fourth Edition Volume 2, Cory, North Carolina, U.S.A., SAS Institute Inc., 1989.
19. Van de Vijver MJ, Peterse JL, Mooi WJ, *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* 1988, **319**, 1239–1245.
20. Clark GM, McGuire WL. Follow-up study of HER-2/neu amplification in primary breast cancer. *Cancer Res* 1991, **51**, 944–948.
21. Berns EMJJ, Klijn JGM, van Putten WLJ, Van Staveren JL, Portengen H, Foekens JA. c.myc amplification is a better prognostic factor than HER-2/neu amplification in primary breast cancer. *Cancer Res* 1992, **52**, 1107–1113.
22. Allred DC, Clark GM, Tandon AK, *et al.* HER-2/neu in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J Clin Oncol* 1992, **10**, 599–605.
23. Neville AM, Bettelheim R, Gelber RD, *et al.* for the International (Ludwig) Breast Cancer Study Group. Factors predicting treatment responsiveness and prognosis in node negative breast cancer. *J Clin Oncol* 1992, **10**, 696–705.
24. Gusterson BA, Gelber RD, Goldhirsch A, *et al.* Prognostic importance of c-erbB-2 expression in breast cancer. *J Clin Oncol* 1992, **10**, 1049–1056.
25. Levine MN, Andrulis I. The HER-2/neu oncogene in breast cancer: so what is new? *J Clin Oncol* 1992, **10**, 1034–1036.
26. Piffanelli A, Dittadi R, Catozzi L, *et al.* Determination of ErbB2 protein in breast cancer tissues by different methods. Relationships with other biological parameters. *Breast Cancer Res Treat* 1996, **37**, 267–276.
27. Toikkanen S, Helin H, Isola J, Joensuu H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30 years follow-up. *J Clin Oncol* 1992, **10**, 1044–1048.
28. Tsuda H, Hirohashi S, Shimosato Y, *et al.* Correlation between histologic grade of malignancy and copy number of c-erbB-2 gene in breast cancer carcinoma. A retrospective analysis of 176 cases. *Cancer* 1990, **65**, 1794–1800.
29. Watanabe T, Fukutomi T, Tsuda H, *et al.* Determination of c-erbB-2 protein in primary breast cancer tissue extract using an enzyme immunoassay. *Jpn J Cancer Res* 1993, **84**, 1279–1286.
30. Adnane J, Gaudray P, Simon MP, Simony TJ, Jeanteur P, Theillet C. Proto-oncogene amplification and human breast tumor phenotype. *Oncogene* 1989, **4**, 1389–1395.
31. Zeillinger R, Kury F, Czerwenka K, *et al.* HER-2 amplification, steroid receptors and epidermal growth factor receptor in primary breast cancer. *Oncogene* 1989, **4**, 109–114.
32. Yamada Y, Yoshimoto M, Murayama Y, *et al.* Association of elevated expression of the c-erbB-2 protein with spread of breast cancer. *Jpn J Cancer Res* 1989, **80**, 1192–1198.
33. Uehara T, Kaneko Y, Kanda N, *et al.* c-erbB-2 and c-erbA-1 (ear-1) gene amplification and c-erbB-2 protein expression in Japanese breast cancers: their relationship to the histology and other disease parameters. *Jpn J Cancer Res* 1990, **81**, 620–624.
34. Berns EM, Klijn JG, Van Staveren JL, Portengen H, Noordegraaf E, Foekens JA. Prevalence of the amplification of the oncogenes c.myc, HER-2/neu and int.2 in one thousand human breast tumours: correlation with steroid receptors. *Eur J Cancer* 1992, **28**, 697–700.
35. Thorpe SM, Christensen JBJ, Rasmussen BB, Rose C. Short recurrence-free survival associated with high estrogen receptor levels in the natural history of postmenopausal primary breast cancer. *Eur J Cancer* 1993, **29A**, 971–977.
36. Sancho-Garnier H, Delarue JC, Mouriesse H, *et al.* Is the negative prognostic value of high estrogen receptor (ER) levels in postmenopausal breast cancer patients due to a modified ER gene product? *Eur J Cancer* 1995, **31A**, 1851–1855.
37. Robertson KW, Reeves JR, Smith G, *et al.* Quantitative estimation of epidermal growth factor receptor and c-erbB-2 in human breast cancer. *Cancer Res* 1996, **56**, 3823–3830.
38. Russell KC, Hung MC. Translational repression of the neu proto-oncogene by estrogen stimulated estrogen receptor. *Cancer Res* 1992, **52**, 6624–6629.
39. McGuire WL, Tandon AK, Allred DC, Chamness GC, Ravdin PM, Clark GM. Treatment decisions in axillary node-negative breast cancer patients. *J Natl Cancer Inst Monogr* 1992, **11**, 173–180.
40. Tsuda H, Hirohoshi S, Shimosato Y, *et al.* Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: hst.1/int.2 and c.erbB.2/ear.1. *Cancer Res* 1989, **49**, 3104–3108.
41. Mittra I, Mac Rae KD. A meta-analysis of reported correlations between prognostic factors in breast cancer: does axillary lymph node metastasis represent biology or chronology? *Eur J Cancer* 1991, **27**, 1574–1583.

Acknowledgements—This work was supported in part by Chiron Diagnostics S.A. Cergy-Pontoise, France. We would like to thank Dr R. Arriagada (I.R.A.M., Santiago, Chile) and H. Mouriesse for helpful discussion, M. Chenuet for preparation of the manuscript, L. Saint-Ange for editing the manuscript and all the clinicians and pathologists of the different centres who actively participated in this study: Centre Paul-Papin, Angers (Dr A. Daver); Fondation Bergonié, Bordeaux (Dr J. Wafflart); Centre François-Baclesse, Caen (Dr J. Goussard); Centre René-Gauducheau, Nantes (Dr G. Ricolleau); Institut Gustave-Roussy, Villejuif (Drs J.C. Delarue, Ph. Terrier, S. Koscielny); Institut Jean-Godinot, Reims (Dr C. Delvincourt).