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PROGNOSTIC VALUE OF THE QUANTITATIVE MEASUREMENT OF THE ONCOPROTEIN P185$^{Her\text{-}2/neu}$ IN A GROUP OF PATIENTS WITH BREAST CANCER AND POSITIVE NODE INVOLVEMENT

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Our study attempts to determine the prognostic value of the quantitative measurement of the oncoprotein p185$^{Her\text{-}2/neu}$ in a group of patients with breast cancer and positive node involvement. In a series of 217 patients with breast cancer and positive nodes in whom the oncoprotein p185 was quantitatively determined by ELISA, we analyzed the clinicopathological variables including age, menopausal status, tumor size, number of affected nodes, type and histology grade and the molecular variables such as the oestrogen and progesterone receptors (ER and PR, respectively), pS2 and Cathepsin D (CD). Using 260 fmol/mg protein as a cut-off point, 18% of the tumors presented as overexpressing p185. The p185 showed no relationship with any of the clinico-pathological variables studied except that its concentration was elevated in ductal and lobular histology types and in the moderate and poorly differentiated histology grades. With a median follow-up of 50 months (range 1–90), the univariate analysis of disease-free survival (DFS) and overall survival (OS) showed that the histology grade, tumor size, the number of infiltrated nodes, the p185 and the ER were the variables associated with the clinical course of the disease in the patients. In the multivariate analysis, however, only the tumor size, number of affected ganglia, the p185 and the ER remained associated with the clinical progression of the disease. The patients with p185 overexpression had a risk, not only of relapse but also death from the disease, of more than twice that of the patients who had normal p185 concentrations. When the p185 was divided into 3 categories based on ±1 × SD above or below the mean, the patients with high and low p185 showed, in the univariate analysis, a similar relationship with DFS but not with OS. In the multivariate analysis, both with the DFS as with the OS, only a high p185 concentration retained its association with the clinical course of the disease in the patients. Our results suggest that by quantitatively determining (using ELISA) the p185 oncoprotein, groups of cancer patients of high risk could be better identified for more effective clinical management.

Key words: *breast cancer; p185 oncoprotein; ELISA*

The HER-2/neu proto-oncogene encodes a 185-kDa trans-membrane glycoprotein (p185) that is a growth factor receptor with tyrosine-kinase activity. The gene amplification or p185 oncoprotein is overexpressed in =30% of cases of human breast cancer[1,2] and has been shown to be associated with a poorer prognosis.[3–6] The HER-2 gene status has also been studied in conjunction with other prognostic biological markers such as positive node involvement, histological grade, size of tumor, oestrogen (ER) and progesterone (PR) steroid receptor, Cathepsin D (Cat D) and pS2.[7–10] The overexpression of p185 has been measured, usually, using immunohistochemical methods and Western blot analysis.[4,11–15] Recently, a quantitative assay[16–18] has been employed for the determination of p185. We have used a standardized ELISA method to quantitatively measure the protein. In the present study we assessed the prognostic value of p185 quantitation in breast cancer patients with lymph node involvement.

MATERIAL AND METHODS

Between December 1990 and March 1996, 217 patients were diagnosed as having invasive breast cancer with ganglia infiltration. The patients' characteristics are summarized in Table I. Median age was 60 years (range 29–80) of whom 61 were premenopausal and 156 were postmenopausal. Infiltrating ductal carcinoma was diagnosed in 186 patients and lobule carcinoma in 25. Staging was T1 in 17 patients, T2 in 146, T3 in 36 and T4 in 18. Tumor status were Grade 1 in 29 patients, Grade 2 in 109 and Grade 3 in 48. Positive ganglia were 1–3 in 123 patients, between 4–9 in 69 and >10 ganglia in 25 patients. Using a cut-off value of 260 fmol/mg protein, the expression of p185 was assigned as normal in 179 patients and overexpressed in 38. Steroid receptor status was RE+ in 158 patients RE− in 59, RP+ in 155, RP− in 62, pS2+ in 124, pS2− in 93, CD+ in 78 and CD− in 139 patients.

Determination of the molecular variables

The total cellular expression of p185 was quantitatively measured using a commercial ELISA kit (Oncogene Science, Uniondale, NY), using 260 fmol/mg protein as a cut-off that we have previously validated.[19,20]

The quantification of the ER and PR were carried out using the method of labeled hormone interchange. A value of 10 fmol/mg protein used as cut-off. The pS2 protein was measured using an immunoradiometric assay (IRMA). A cut-off point of 2 ng/mg protein was used. Quantitative measurement of the CD was with IRMA. The cut-off point was 75 pmol/mg protein.

Statistical analyses

To define the values of the different quantitative variable we used the frequency distribution, central tendency measures of mean and median; of dispersion; SEM; SD and range, and test of normality (Kolmogorov-Smirnov test).

The statistical evaluations of the dichotomized qualitative variables were by using contingency tables and the χ^2 test and Fisher's exact test.

In the univariate analysis of disease free survival and of overall survival, we used the method of Kaplan-Meier and the log-rank test for the analysis of differences between the curves and the regression model of Cox. In the multivariate analysis of disease

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TABLE I – CLINICAL CHARACTERISTICS OF THE PATIENTS ENROLLED IN THE STUDY

Features of the patients	
Number of patients	217 (100%)
Mean age	60 years (range 29–90)
Pre/perimenopausal	61 (28%)
Postmenopausal	156 (72%)
Histological type	
Ductal	186 (86%)
Lobular	25 (12%)
Other	6 (3%)
Histological grade	
G1	9 (16%)
G2	109 (59%)
G3	48 (26%)
Size	
T1	17 (8%)
T2	146 (67%)
T3	36 (17%)
T4	18 (8%)
Nodes	
N1	158 (72.8%)
N2	59 (27.2%)
N3	0 (0%)
Number of positive nodes	
1–3	123 (56.7%)
4–9	69 (31.8%)
>1	25 (11.5%)
Receptors	
ER+	158 (72.8%)
ER−	59 (27.2%)
PR+	155 (71.4%)
PR−	62 (28.6%)
pS2	
pS2+	124 (57.1%)
pS2−	93 (42.9%)
Catepsin D	
Cat D+	78 (35.9%)
Cat D−	139 (64.1%)

free survival and overall survival we used Cox0s regression model. Statistical significance was established at $p < 0.05$.

RESULTS

Between December 1990 and March 1996, 217 patients were diagnosed as having invasive breast cancer or node infiltration. The levels of p185 were significantly more elevated ($p = 0.03$) in those with ductal and lobular types (median 120 and 128, respectively) than the other histologic types (median 55). With a median follow-up of 50 months (range 1–90), the disease-free survival (DFS) was 60% and the overall survival (OS) was 67%. There were 63 relapses (29%) and 47 (22%) deaths. Significant differences were observed in the DFS and OS curves between patients when segregated with respect to: grade of tumor differentiation; $p = 0.0026$ and $p = 0.005$; DFS and OS, respectively; log-rank test); tumor size; $p < 0.0001$ and $p < 0.0001$; DFS and OS, respectively; log-rank test); number of positive ganglia; $p < .001$ and $p < 0.0001$; DFS and OS, respectively; log- rank test); RE; $p = 0.002$ and $p = 0.0004$; DFS and OS, respectively; log-rank test); p185 overexpression; $p = 0.003$ and $p = 0.008$; DFS and OS, respectively; log-rank test). The patients with p185 overexpression had a poorer DFS than the patients who did not have overexpression within the group of patients with ER+/PR+ tumors; $p = 0.04$; log- rank test). Similarly, in the groups of patients with ER−/PR−, the patients with p185 overexpression had a poorer DFS that the patients who did not have p185 overexpression; $p = 0.04$; log-rank test). In the group of patients with ER−/PR− tumors, however, the patients with p185 overexpression had a similar OS compared to the patients who did not have p185 overexpression; $p = 0.4$; log-rank test). When the tumors were segregated into 3 groups using the concentration distribution of p185 expression, (the mean p185 value was 160 fmol/mg p., range 0–2513 fmol/mg p; range of p185 values for each group was low = 0–<40 fmol/mg p., normal = 40–<350 fmol/mg p. and high = 350 fmol/mg p.) (Fig. 1), it was observed that the patients above and below the mean ± 1 × SD values had poorer DFS and OS than the patients with normal p185 expression (Figs. 2,3); $p = 0.003$ and $p = 0.01$; DFS and OS, respectively (log-rank test).

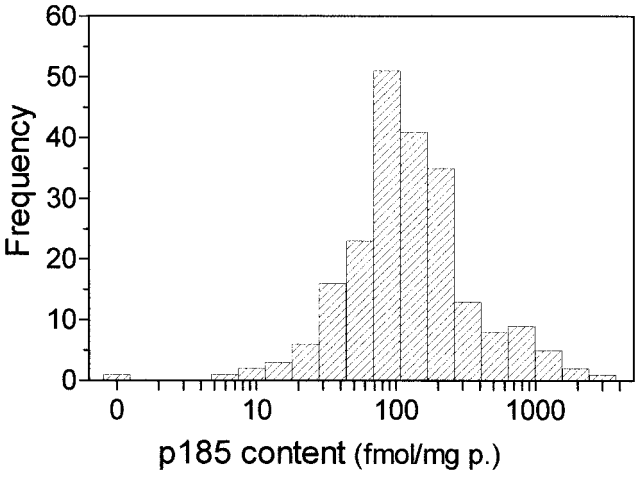

FIGURE 1 – Frequency distribution of p185 values, logarithmically transformed, in 217 breast-tumor extracts.

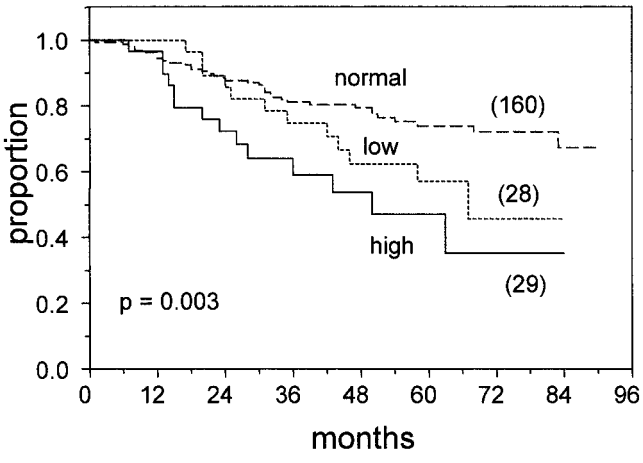

FIGURE 2 – Disease-free survival (DFS) segregated with respect to the 3 categories of high, normal or low p185 expression based on the mean ± 1 × SD of the distribution.

Univariate and multivariate analyses of DFS and OS were carried out using the method of Kaplan-Meier, the log-rank test and Cox's proportional risk regression model. The results are summarized in Tables II–V. Histology grade, tumor size, number of ganglia affected, p185 expression and ER status were significantly associated with DFS as well as OS. Cox's method demonstrated that, for DFS, when the p185 expression was divided into 3 categories with respect to the mean ± 1 × SD, the patients above and below the normal levels had a poorer clinical outcome that those within the normal expression of p185. Although there was a trend toward an association between low p185 expression and relapse, this did not reach statistical significance ($p = 0.07$). With respect to OS, patients with high levels of p185 expression had a poorer OS than the patients with tumors having normal p185 levels. No significant differences were observed in the clinical

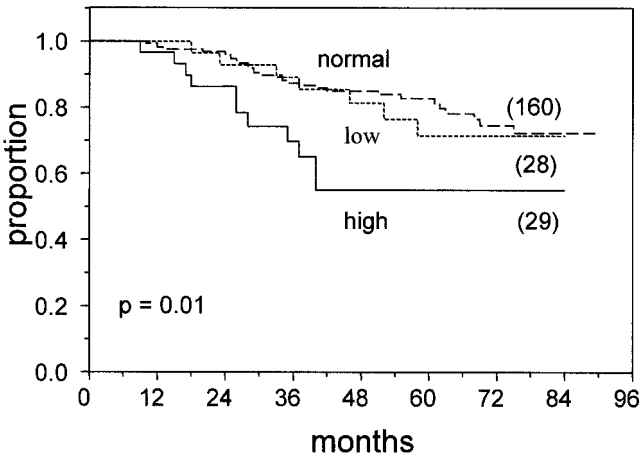

FIGURE 3 – Overall survival (OS) segregated with respect to the 3 categories of high, normal and low p185 expression based on the mean ± 1 × SD of the distribution.

course of the disease in the patients with low and normal p185 levels. The results of the multivariate analysis of DFS and OS showed that the inclusion or exclusion of the histology type, the PR, the pS2 and the CD did not modify the final model nor the levels of statistical significance. The results are summarized in Tables II–V. The inclusion or exclusion of the histology grade did not affect the variables that had been included in the model, although the level of statistical significance became slightly modified. The age of the patients and the menopausal status were always included in the analyses as possible confounding variables.

DISCUSSION

In the present study using 260 fmol/mg protein as cut-off, we observed that 18% of the tumors had p185 overexpression. This percentage was within the range of 10–40% of the cases observed by other authors[4,7,21] using gene amplification or overexpression of the p185.

The possibility has been suggested in recent studies that not only the tumors with overexpression p185 have an altered clinical course but so also do those in which the expression of the p185 is low.[14,17] To acquire more information that could be clinically useful, we divided the data into 3 patient subgroups with respect to p185 expression using the mean ± 1 × SD as cut-off. According to this criterion, 13% of the tumors contained low levels of p185, 74% had normal levels and 13% had high levels. Steroid receptor status was assessed using the labeled hormone exchange assay. This is a widely used procedure or measuring tumor markers. The cut-off of 10 fmol/mg protein used to transform the continuous values into binomial distributions is the procedure selected by the majority of tumor marker laboratories. In our series of patients, 73% of the tumors were ER+ and 72% were PR+. These percentages are within the range of published data.

We used an immunoradiometric assay to measure the levels of the pS2 and CD. The data obtained (51% for pS2 positive and 36% for the CD positive) are in accord with the published literature.[22,23]

As with the majority of authors, we did not observe any association between the p185 and the age of the patients at the time of diagnosis (dichotomized around the median) and the menopausal status. We observed an elevated p185 content in the moderately- and poorly-differentiated tumors but we did not observe any relationship between the p185 and the tumor size nor between the p185 and the number of infiltrated nodes.[24–27] In our series of patients, with a mean of follow-up of 4 years, relapse occurred in 29% of the patients and the disease-free survival (DFS) at 7 years was estimated as 60%. These values are similar to that described by the majority of studies.[24,25,28–30] Consistent with this lower number of relapses, death caused by the disease was only 22% of the patients and the probability of overall survival (OS) at 7 years was estimated as 69%. Because the clinico-pathological characteristics of our series of patients are similar to the majority of those described in the literature, it is probable that this better clinical outcome was due to the good selection and more appropriate application of the post-surgery adjuvant therapy.

Of the relapses observed, the majority were distant metastases and only 3 regional metastases and 2 local relapses were encountered. With respect to the distant metastases, and in accord with that described in other series of patients,[31,32] the anatomic sites most affected were bones in 26 patients, lung in 24 and liver in 15 patients.

Analysis of the DFS curves show that the patients with the p185 overexpression have a poorer DFS than patients with normal p185 expression in terms of relapse (45% *vs.* 26%, respectively; $p = 0.003$) and estimated probability of DFS at 7 years (22% *vs.* 68%, respectively; $p = 0{,}003$. The data in the literature from the more-important studies (containing >100 patients and using non-quantitative techniques for the measurement of the oncoprotein) are almost unanimous in associating the amplification or overexpression of the HER-2/neu with shorter DFS.[4,7,33–36] Only Thor *et al.*[37] did not observe this association whereas Borg *et al.*[38] encountered an association with amplification but not with overexpression. Our results coincide with those studies in which an ELISA method similar to our study was used for the quantitative measurement of the p185 oncoprotein.[14,17,39]

Analysis of the OS curves in our patients showed that the patients who had tumors overexpressing p185 had a shorter OS than those with tumors with normal p185 expression (45% *vs.* 19%, respectively; $p = 0.008$) and a poorer OS at 7 years (42% for the patients with positive p185 compared to 74% for those with negative p185; $p = 0.008$. These results coincide with 9 of the 10 most important references in the literature[1,8,11,35–37,40-42] in which the amplification or overexpression of HER-2/neu was estimated by semi-quantitative procedures. Among the more important studies, only Thor *et al.*[37] observed an absence of association between the HER-2/neu status and the OS. In a single article published[42] on the relationship between the quantitatively-measured p185 and the OS, a result similar to ours was obtained.

In our study population, p185 overexpression was associated with a shorter DFS, not only in the groups of patients with ER+PR+ but also in the group ER!PR! despite the numbers of patients in the latter group being relatively small (29 patients). Similarly, overexpression of the oncoprotein was associated with a marginally poorer OS in the group of patients with ER+/PR+. Earlier, Dati *et al.*[43] had published similar results to ours in PR+ patients.

When the levels of p185 were divided into 3 groups based on the values of the mean ±1 × SD, the DFS analyses using Kaplan-Meier survival curves and with the log-rank test, we observed that the patients with low p185 (mean ± 1 × SD) and the patients with high p185 (mean ± 1 × SD) had shorter DFS than those patients with normal p185 (*i.e.*, levels within the mean ± 1 × SD; $p = 0.003$). There were relapses in 43% of the patients with low p185 and in 48% of the patients with high p185 compared to only 23% in the patients with normal p185. The estimated DFS at 7 years was 46%, 35% and 67% (low, high and normal p185, respectively). These results, although somewhat surprising, coincide with those obtained by 2 other studies in which the p185 was measured quantitatively using ELISA in a system similar to our own.[14,17] According to those results, the tumors with low levels of p185 were characterized by having low ER and PR and that was the explanation for the poor clinical outcomes for the patients with these tumors. Another hypothesis that could explain this phenomenon was proposed by Koscielny *et al.*[17] who suggested a possible interaction between various oncogenes, such that the absence of expression of one of them is counterbalanced by the overexpres-

TABLE II – DISEASE FREE SURVIVAL, UNIVARIATE AND MULTIVARIATE ANALYSES

Variables	Kaplan-Meier *p*	Cox *p*	Cox RR (CI 95%)[1]
Age	0.07	0.08	
Menopausal status	0.2	0.2	
Histological type	0.7	0.7	
Histological grade	**0.003**	**0.004**	
G2 *vs.* G1		0.3	
G3 *vs.* G1		**0.01**	3.8 (1.3–11.0)
Tumour size	**0.0000**	**0.0001**	
T2 *vs.* T1		0.5	
T3/T4 *vs.* T1		**0.03**	5.0 (1.2–21.0)
Positive nodes	**0.0001**	**0.0003**	
4–9 *vs.* 1–3		**0.008**	2.1 (1.2–3.7)
>9 *vs.* 1–3		**0.0001**	4.0 (2.0–7.8)
Molecular variables			
P185	**0.003**	**0.004**	1.5 (1.1–2.0)
ER	**0.002**	**0.003**	1.5 (1.1–1.9)
PR	0.7	0.7	
PS2	0.6	0.8	
Catepsin D	0.9	0.8	
P185			
Overexpressed *vs.* normal	6.7[2]	0.009	2.1 (1.2–3.7)
Oestrogen receptor			
Negative *vs.* positive	8.3[2]	0.004	2.1 (1.3–3.5)
Tumour size			
T3/T4 *vs.* T1/T2	11.8[2]	0.0006	2.5 (1.5–6.1)
Nodes affected	9.7[2]	0.01	
4–9 *vs.* 1–3	3.9[2]	<0.05	1.8 (1.007–2.1)
>9 *vs.* 1–3	9.2[2]	0.002	3.0 (1.5–6.1)

[1]RR (95% CI), relative risk (95% confidence interval).–[2]Wald.

TABLE III – OVERALL SURVIVAL, UNIVARIATE AND MULTIVARIATE ANALYSES

Variables	Kaplan-Meier *p*	Cox *p*	Cox RR (95% CI)[1]
Size	0.2	0.2	
Menopausal status	0.6	0.6	
Histological type	0.7	0.7	
Histological grade	**0.005**	**0.009**	
G2 *vs.* G1		0.5	
G3 *vs.* G1		**0.03**	3.8 (1.1–12.7)
Tumour size	**0.0000**	**0.0001**	
T2 *vs.* T1		0.4	
T3/T4 *vs.* T1		**<0.05**	7.4 (1.003–54.9)
Affected nodes	**0.0000**	**0.0000**	
4–9 *vs.* 1–3		**0.001**	3.2 (1.6–6.3)
>9 *vs.* 1–3		**0.0000**	7.5 (3.5–16.3)
Molecular variables			
P185	**0.008**	**0.01**	2.3 (1.2–4.4)
ER	**0.0004**	**0.0008**	2.7 (1.5–4.7)
PR	0.6	0.6	
PS2	0.6	0.6	
Catepsin D	0.9	0.9	
P185			
Overexpresed *vs.* normal	6.7[2]	0.009	2.4 (1.2–4.6)
Oestrogen receptors			
Negative *vs.* positive	10.2[2]	0.001	2.6 (1.4–4.7)
Tumour size			
T3/T4 *vs.* T1/T2	8.8[2]	0.003	2.5 (1.5–6.1)
Affected nodes	17.7[2]	0.0001	
4–9 *vs.* 1–3	8.2[2]	0.004	2.8 (1.4–5.7)
>9 *vs.* 1–3	17.3[2]	0.0000	5.7 (2.5–13.0)

[1]RR (95% CI), relative risk (95% confidence interval).–[2]Wald.

sion of another and, within such a context, overexpression per se is not the only factor *i.e.*, all deviations from the "normal" expression, whether high or low, need to be considered as determining factors.

It needs to be highlighted, nevertheless, that the univariate analysis carried out using the method of Cox showed that only the patients with high p185 had a DFS significantly shorter that the patients with normal p185 and that the poor prognosis of the patients with low p185 approached, but did not achieve, statistical significance ($p = 0.07$). These results do not, however, invalidate the above commentaries, and could be of considerable importance in the prognosis of these patients.

Compared to the DFS analysis, the OS curves showed that the patients with low p185 had a prognosis similar to those patients

TABLE IV – DISEASE FREE SURVIVAL AND P185, UNIVARIATE AND MULTIVARIATE ANALYSIS

Variable	Wald	Kaplan-Meier *p*	Cox *p*	Cox RR (95% CI)[1]
P185 (mean ± 1 × SD)		**0.003**	**0.007**	
Low *vs.* normal			0.078	
High *vs.* normal			**0.001**	2.8 (1.5–5.1)
P185	5.5		0.05	
High *vs.* normal	5.1		0.02	2.1 (1.1–3.9)
Oestrogen receptor				
Negative *vs.* positive	8.3		0.004	2.2 (1.3–3.6)
Tumour size				
T3/T4 *vs.* T1/T2	11.5		0.0007	2.5 (1.5–4.1)
Nodes affected	8.0		0.01	
4–9 *vs.* 1–3	3.5		0.06	1.7 (0.97–3.1)
>9 *vs.* 1–3	9.2		0.006	2.9 (1.4–6.0)

[1]RR (95% CI), relative risk (95% conficence interval).

TABLE V – OVERALL SURVIVAL AND P185, UNIVARIATE AND MULTIVARIATE ANALYSIS

Variable	Wald	Kaplan-Meier *p*	Cox *p*	Cox RR (95% CI)[1]
P185		**0.01**	**0.03**	
Low *vs.* normal			0.63	
High *vs.* normal			**0.005**	2.7 (1.4–5.5)
P185	7.2		0.03	
High *vs.* normal	3.6		0.05	2.0 (1.0–4.1)
Oestrogen receptors				
Negative *vs.* positive	12.3		0.0005	3.0 (1.6–5.4)
Tumour size				
T3/T4 *vs.* T1/T2				
Affected nodes	18.1		0.01	
4–9 *vs.* 1–3	8.6		0.003	2.9 (1.4–6.0)
>9 *vs.* 1–3	17.7		0.0000	6.1 (2.6–14.5)

[1]RR (95% CI), relative risk (95% confidence interval).

with a normal p185 and that the OS of the patients with high p185 were significantly worse than that of the other 2 groups of patients. None of the authors cited above[14,17] studied the influence of stratified p185 on the OS. It could be inferred from our data that the high as well as the low levels of p185 predispose to an earlier patient relapse whereas only the high levels of p185 imply a poorer overall survival. This phenomenon may be related to different post-relapse treatment response of tumors with different levels of p185 expression.

Of all the variables studied, the only ones that were significantly associated with DFS as well as with OS, were the levels of p185 expression, tumor size, number of positive ganglia and positive steroid receptor status.

Among the published studies in which the HER-2/neu status was semi-quantitatively measured, a slight majority[4,7,33,41,44,45] indicate a significant association between the oncogene status and DFS, whereas the others[34,38,46] do not describe any such association. Only in 2 studies in which the p185 was measured quantitatively and in which a multivariate analysis of DFS carried out, was there described a significant influence of the levels of the oncoprotein on the clinical outcome in the patients.[14,39]

Conversely, the majority of the authors highlight the absence of association between the HER-2/neu status and OS1,[34–38,41] and only a few describe a significant association.[4,7,44] In none of these studies was the p185 expression determined quantitatively and multivariate analysis of the OS carried out.

As we have demonstrated, our study could be the pioneer in demonstrating (using multivariate analysis) the association between overexpression of the p185 (quantitatively determined) and the shorter DFS and OS in patients with breast cancer and positive node involvement. When stratified with respect to the distribution of p185 expression, our results indicated that only the tumors with high p185 expression are associated with a poorer DFS and OS. These results contrast with those of Dittadi *et al.*[14] that indicated that patients with tumors with low p185 expression also need to be considered of high risk of early relapse. No comparisons with respect to OS may be made because this aspect has not been explored in the literature to date.

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