Strong Correlation between Results of Fluorescent *In Situ* Hybridization and Immunohistochemistry for the Assessment of the ERBB2 (HER-2/*neu*) Gene Status in Breast Carcinoma

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ERBB2 (HER-2/*neu*) amplification and/or overexpression are associated with poor prognosis in node-positive breast carcinoma. Its prognostic value in node-negative cases and its predictive value for response to chemotherapy remain controversial. This may be related to the use of molecular methods, which are sensitive to dilution of tumor material by normal cells, or the use of nonstandardized immunohistochemistry (IHC) procedures, for the determination of the ERBB2 gene status. In addition, new therapeutic approaches that target the cells overexpressing ERBB2 are under development. These perspectives necessitate a reliable evaluation of the status of ERBB2 in individual tumors before the application of specific therapeutic strategies. Fluorescent *in situ* hybridization (FISH) and IHC allow the evaluation of the ERBB2 status specifically in tumor cells on archival material. We have analyzed a series of 100 invasive ductal breast carcinomas without lymph node invasion both by IHC, using the CB11 monoclonal antibody and a sensitive Auidin Biotin Complex (ABC) immunodetection system, and by FISH, using the Oncor Inform HER-2/*neu* (ERBB2) gene amplification detection system as reference technique. Complete concordance between the results of FISH and IHC was seen in 98% of the cases. ERBB2 amplification (more than four signals per nucleus) was observed in 12 of the 100 cases, and all but one showed an overexpression of the protein (membrane staining) by IHC. Conversely, ERBB2 expression was present in one case without gene amplification. In conclusion, ERBB2 overexpression detected by IHC is highly correlated to gene amplification detected by FISH. Thus, under standardized conditions, IHC is a reliable and economical test to assess the ERBB2 status in tumors. The use of FISH could be limited to the verification of the status of tumors displaying a weak membrane immunostaining.

KEY WORDS: Breast carcinoma; c-erbB2; ERBB2; HER-2/neu; fluorescent *in situ* hybridization (FISH); Immunohistochemistry; Node negative.

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The ERBB2 (HER2/*neu*) oncogene, a member of the type I growth factor receptor gene family, located on the long arm of chromosome 17, is found amplified and/or overexpressed in about 20 to 30% of primary breast carcinomas (1, 2). Several works suggest that this amplification/overexpression is associated with poor prognosis in breast carcinoma, especially in node positive cases (1–5; for review, see 6, 7) and that it could be a marker of reduced response to chemotherapy (3, 4, 8, 9; for review, 6, 7). Conversely, whereas some authors provided data suggesting that ERBB2 expression was a marker of preferential response to anthracycline-containing regimens (10–11), others found no significant correlation (12–14). The value of ERBB2 amplification/overexpression as an independent prognostic factor in node-negative cases remains controversial (8, 15, 16; for review, see 6). In addition, new therapeutic approaches that target the cells overexpressing ERBB2, based on monoclonal antibodies (17–19) and on antisense oligonucleotides (20), are under development. These perspectives necessitate an accurate knowledge of the ERBB2 status in individual tumors before the application of specific therapeutic strategies. ERBB2 gene status has been assessed mainly by Southern or dot blot, and by the polymerase chain reaction.

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Its overexpression has been detected by immunohistochemistry (IHC) in the majority of the cases, and by Western and Northern blot (for review, 6, 7). The range of positivity found in the various studies seems to be rather large: 5 to 55% for the amplification, and 10 to 55% for the overexpression (6). Indeed, results of molecular methods may be affected by the dilution of tumor material by normal cells and by the determination of the threshold defining the existence of an amplification or an overexpression (2, 7, 21). Recent technical developments have made possible the detection of the ERBB2 gene in tissue sections, touch preparations, and fine-needle aspirates using fluorescent *in situ* hybridization (FISH) (16, 22–32). The most reliable method to routinely evaluate the status of ERBB2 in prospective and archival material remains to be determined. Both FISH and IHC allow the evaluation of the status of ERBB2 on a cell-by-cell basis in archival material Therefore, they should be regarded as methods of choice for this assay. However, correlative results of these two approaches on the same samples are not yet amply available (26–32). To further assess the correlation between amplification and expression of this gene, and to evaluate ERBB2 in node-negative cases, we studied a series of 100 node-negative breast carcinomas, using both FISH and IHC on tissue sections from paraffin-embedded blocks.

MATERIALS AND METHODS

Patient Material

The samples analyzed were obtained from patients with invasive ductal carcinoma without lymph node involvement, who had undergone primary surgery only. Clinicopathological data of the patients and tumor samples studied are reported in Table 1. Tumor material consisted of 100 paraffin-embedded specimens fixed in AFA (75% ethanol 100°/5% acetic acid/2% commercial formalin/18% deionized water). For each case, the histology of the sample analyzed was verified on a hydroxyethyl starch-stained preparation, and areas showing invasive carcinoma were identified. The status of ERBB2 was independently assayed by FISH and IHC on two consecutive 4-μm tissue sections.

Table 1. Clinicopathological data of 100 cases of node-negative breast ductal carcinoma.

		Number of cases
Patient age (years)	<50	32
	>50	68
Tumor size (mm)	<20	61
	>20	39
Histoprognostic index (SBR)	I	18
	II	63
	III	19

Immunohistochemistry

After deparaffinization and rehydration of the sections, endogenous peroxidases were inhibited by a 30-min incubation in 0.3% H_2O_2 in deionized water. All further steps were performed using an automated system. Tissue sections were incubated with the monoclonal anti c-erbB-2 oncoprotein NCL-CB11 (Novocastra Laboratories, Newcastle-on-Tyne, UK), diluted 1/400, for 1 h at 20°C, without antigen retrieval. The CB11 antibody was selected among two other antibodies assayed, A485 (Dako, Carpinteria, CA, USA), and Tab250 (Zymed, San Francisco, CA, USA), for its higher sensitivity and specificity in our experimental conditions. Immunodetection was achieved using the Vectastain Elite ABC peroxidase mouse IgG kit (Vector Laboratories, Burlingame, CA, USA), following the instructions given by the supplier. Then, slides were incubated with 3,3′-diaminobenzidine for 5 min and counterstained with Mayer's hematoxylin. A positive control was included in each processed series of slides. Preparations were analyzed independently by two investigators. Under these conditions, normal epithelial cells were not stained, and thus represented an internal negative control. The percentage of invasive carcinomatous cells showing a membranous staining, and the intensity of the staining (absent, weak, or strong) was recorded. The analysis lead to classify the tumors into three groups: cases without detectable staining in tumor cells [*i.e.*, no ERBB2 overexpression (0)]; cases showing weak staining in the majority (more than 60%) of tumor cells [*i.e.*, weak ERBB2 overexpression (+)]; cases showing strong staining in the majority (more than 60%) of the tumor cells [*i.e.*, strong ERBB2 overexpression (++)]. Cases with only a minimal percentage of positive tumor cells were not observed.

FISH

FISH experiments were carried out using the Oncor Inform HER-2/neu (ERBB2) gene amplification detection system (Ventana Medical Systems, Tucson, AZ, USA), following the instructions given by the supplier. Briefly, deparaffinized slides were first treated with a protein-digesting enzyme at 37°C for 40 min. The preparations were denatured in 70% formamide/2× standard saline citrate (SSC), pH 7, at 75°C for 8 min. The mixture containing a biotinylated HER-2/*neu* DNA probe was applied to the denatured preparations, and slides were incubated for 15 hours at 37°C. The slides were washed in 50% formamide/2× SSC, pH 7, at 43°C for 15 min, then

in 2× SSC, pH 7, at 37°C, for 10 min. Detection was achieved using fluorescein-labeled avidin, then an antiavidin antibody, followed by a second layer of fluorescein-labeled avidin. The nuclei were counterstained using a 4,6-DiAmidino-2-pheylIndole (DAPI)/antifade solution. The slides were viewed with an epifluorescence Leica DMRB microscope fitted with DAPI and double-band DAPI/fluorescein isothiocyanate filters. Images were captured using a Quantics digital camera (Photometrics, Tucson, AZ, USA) and Quips FISH imaging software (Vysis, Downers Grove, IL, USA). The number of fluorescein signals was counted in 20 nuclei of invasive tumor cells, referring to the hydroxyethyl starch-staining when necessary, in two distant areas of the section. For each tumor, the mean number of signals per nucleus was determined. Tumors showing a score of more than four signals per nucleus were considered to have ERBB2 amplification. In two cases in which a discordance between the results of FISH and IHC had been observed, a dual color FISH of ERBB2 and centromere of chromosome 17 (PathVysion HER-2 kit, Vysis) was performed, according to the instructions of the supplier.

Statistical Analysis

The concordance between the two methods was assessed using the kappa test. The sensitivity and specificity of IHC was evaluated, using dichotomous values (+, −), taking FISH as reference method.

RESULTS

The majority of the patients analyzed (68%) were older than 50 years. The large majority of the tumors were less than 20 mm in diameter (61%), and SBR grade II (63%).

The results of the ERBB2 status as determined by FISH and IHC are given in Table 2, and representative pictures of FISH and IHC are shown in Figures 1 and 2, respectively.

FISH

ERBB2 amplification was observed with the use of FISH in 12 of the 100 cases analyzed. In cases with an amplification, fluorescent signals were often arranged in clusters (Fig. 1B). In nine of these cases, the mean number of signals per nucleus was higher than 20. In the three other cases, the mean numbers were 12.5, 8.8, and 5.2. In this last case, the existence of an amplification was checked by dual color FISH with a centromeric probe of chromosome 17 in addition to the ERBB2 probe: tumor cells had a mean number of 1.7 signals corresponding to chromosome 17 (Fig. 1C).

Table 2. Comparison between the status of ERBB2 gene as determined by FISH, and its expression determined by IHC in 100 cases of node-negative breast carcinomas.

		ERBB2 expression (IHC)			
		0	+	++	Total
ERBB2 status	NA	87	1	0	88
(FISH)	A	1	3	8	12
Total		88	12		100

A, amplified; NA, not amplified; ++, strong; +, weak; 0, not detected.

Immunohistochemistry

ERBB2 overexpression was detected by IHC in 12 of the 100 cases analyzed. It was scored as strong (Fig. 2) in eight cases and weak in four cases. No discrepancies were observed between the two pathologists in the identification of positive cases. In our experimental conditions, the percentage of labeled tumor cells in positive cases always exceeded 60%, and the intensity of the staining could be readily scored according to one of the two defined categories (weak or strong). A concordance between amplification and detectable expression of the ERBB2 gene was found in 98 of the 100 cases examined ($\kappa = 0, 90$; $P < 0.0001$). Sensitivity of IHC, with FISH as the reference technique, was 92% (86 to 97%, 95% confidence interval), and specificity was 99% (97 to 100%, 95% confidence interval). Positive immunostaining was observed in 11 of the 12 cases with gene amplification. One case with weak ERBB2 expression showed no evidence of gene amplification. This case was checked by dual color FISH: the majority of tumor cells had two ERBB2 and chromosome 17 signals. Conversely, one case negative for ERBB2 expression by IHC had gene amplification (Table 2). This tumor showed a mean number of 8.8 ERBB2 gene copies per nucleus.

DISCUSSION

Reliable biological markers with prognostic and predictive significance are strongly needed for therapeutic decisions in breast carcinoma, in particular for the recognition of the 20 to 30% of lymph node-negative patients who will experience recurrence and for predicting the response to chemotherapy in node-positive patients. More than 10 years after the first report by Slamon *et al.* (1) showing the existence of a correlation between ERBB2 amplification and poor prognosis in breast carcinoma, the role of ERBB2 amplification/overexpression as an independent prognostic marker remains controversial, especially in node negative cases (3, 5, 8, 15, 16; for review, see 6, 7). Similarly, its predictive value regarding tumor response to treatment is still unclear (9–14; for review, see 6, 7). A part of the uncertainty

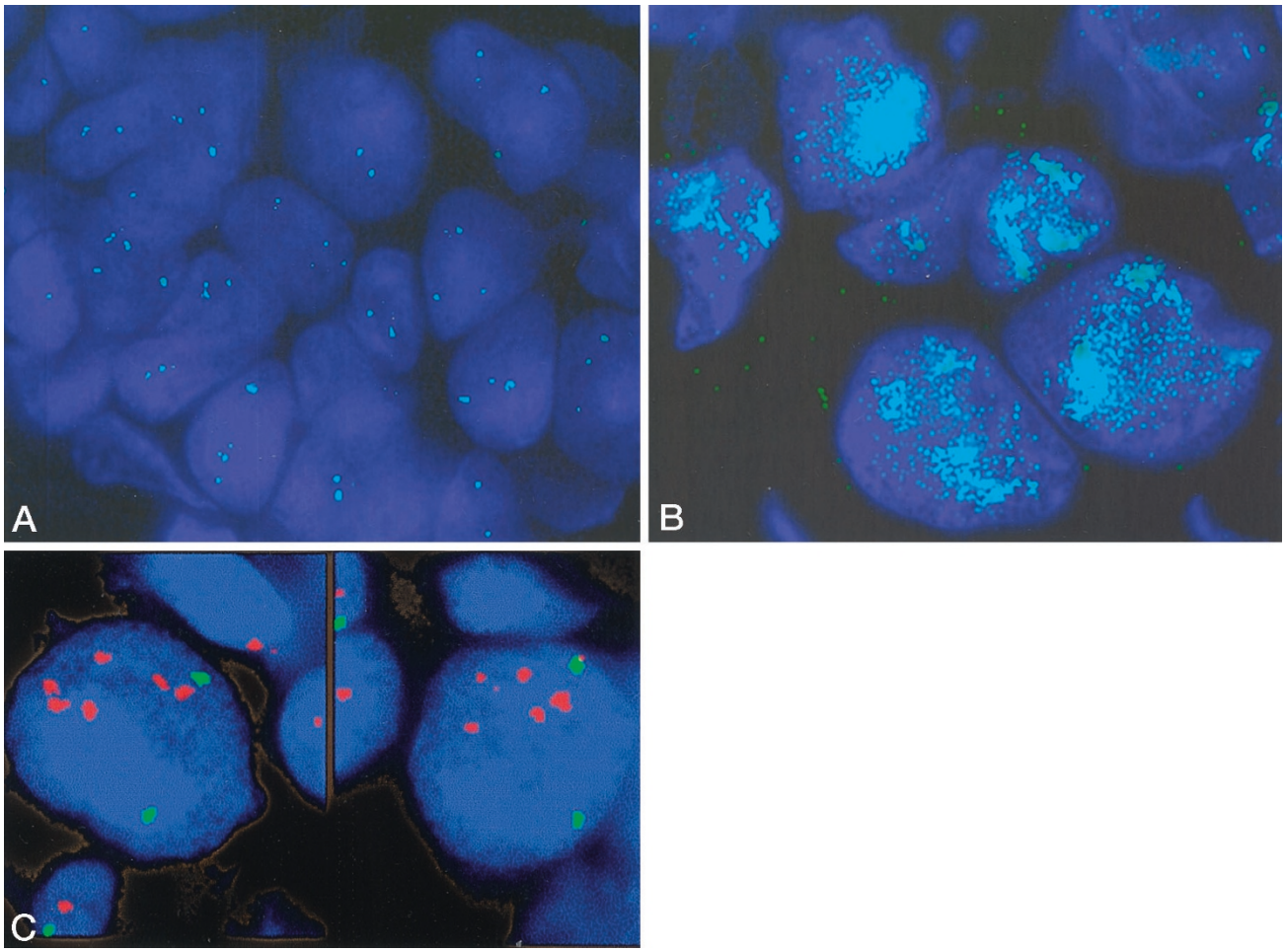

FIGURE 1. FISH of ERBB2 probe on paraffin-embedded tissue sections of three cases of breast carcinoma. **A**, tumor without amplification. A mean number of two signals per nucleus is observed. **B**, tumor with a high level amplification. Presence of numerous signals, arranged in clusters. **C**, tumor with the lowest amplification (dual color FISH): presence of five to six signals of the ERBB2 probe (***red***) and two centromeres of chromosome 17 (***green***).

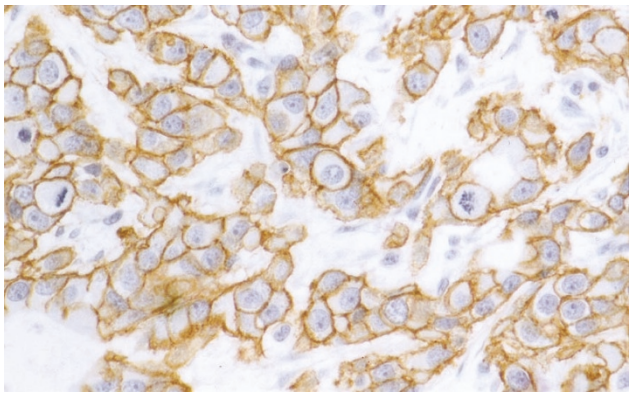

FIGURE 2. Immunohistochemistry using the CB11 anti-c-erbB-2 antibody and ABC technique on a paraffin-embedded tissue section of a breast carcinoma. This tumor shows a strong expression of the protein (membrane labeling).

in establishing the prognostic and predictive value of ERBB2 may be related to technical difficulties in assessing the status of the gene by molecular techniques. In earlier studies, the status of ERBB2 was evaluated by Southern, Northern, or Western blot, and, more recently, by polymerase chain reaction. Results of these methods may be affected by the dilution of tumor material by normal cells (2, 21). These drawbacks have prompted the development of *in situ* techniques, FISH and IHC, which enable the recognition of the marker of interest in tumor cells only. Slamon *et al.* (2) have shown the existence of a direct correlation between amplification and overexpression of ERBB2. However, Press *et al.* (32) have stressed the variability of the sensitivity of HER-2/*neu* antibodies as potential source of errors in the assessment of the expression of the gene. The present controversy about the reliability of a U. S. Food and Drug Administration-approved HER-2/*neu* IHC test (33–34) illustrates that this issue remains incompletely solved. Yet, the recently introduced therapeutic approaches, based on monoclonal antibodies (17–19) and on antisense oligonucleotides (20), that target tumor cells overexpressing ERBB2 require an accurate assessment of the status of the gene before inclusion of patients in such protocols.

These reasons mandate the necessity to calibrate IHC with the aid of a reference technique, which could be FISH. Farabegoli *et al.* (28), obtained a correlation of only 72% between FISH and IHC in a study of 79 primary invasive breast carcinoma samples, because of the observation of an excess of immunopositive cases without amplification. Similarly, Jimenez *et al.* (32) observed, in a comparative study of 41 cases, a proportion of 3 to 15% of cases, according to the antibody used, showing moderate immunostaining without amplification. Jacobs *et al.* (29), in a study of 100 consecutive paraffin-embedded breast carcinomas samples, demonstrated a concordance of 92% between the results of FISH and IHC, using the Oncor Inform kit and the Dako A485 polyclonal antibody. These results led the authors to recommend the use of IHC, under standardized conditions, as a routine technique for the evaluation of the ERBB2 status. Only one large study on node-negative breast carcinomas, using a combined approach by FISH and IHC on touch preparations has been reported (25). The authors used a HER-2/*neu* SpectrumOrange probe (Vysis) and a polyclonal anti-c-erbB-2 antibody (Dako). They observed a rate of 16% amplified cases and a concordance in 92% of the cases for ERBB2 gene amplification detected by FISH and protein overexpression detected by IHC.

We have observed a rate of 12% for ERBB2-amplified tumors in our series of node-negative breast carcinomas. Nine of the 12 tumors with ERBB2 amplification showed more than 20 copies of the gene per nucleus. Fluorescent signals were often arranged in clusters (Fig. 1B), corresponding to homogeneously staining regions observed in metaphase chromosomes of breast carcinomas (35). In our series, a concordance of 98% was observed between the gene status and its protein expression detected by IHC. One case with amplification did not display protein overexpression by IHC. Interestingly, this case showed a low mean number of signals per nucleus (8.8 per nucleus). Similar finding has been noticed by Jacobs *et al.* (29). The existence of cases with low-level amplification leads us to recommend the use of dual-color FISH with a centromeric probe of chromosome 17 to distinguish polysomy 17 from true ERBB2 amplification. In one case, a weak overexpression was observed without gene amplification. Comparing the results of the two techniques shows that if membrane labeling is present in a cluster of tumor cells, at any intensity, a gene amplification may exist. However, some rare cases with low intensity staining (+) may be unamplified. Such expression may be related to other mechanisms of activation of the ERBB2 gene (2, 29).

In our experience, the factors that optimally determined the results of IHC and gave a good correlation with FISH were the use of an automated protocol, the selection of the monoclonal antibody CB11, its appropriate concentration to yield no staining in normal epithelial cells, and the use of a sensitive ABC detection system. In conclusion, this study demonstrates that, IHC can reliably detect ERBB2-amplified tumors if rigorous conditions of sensitivity and standardization are applied. The use of FISH could therefore be limited to the control of the rare cases showing low protein expression by IHC.

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