The Combination of *In Situ* Hybridization and Immunohistochemical Analysis: An Evaluation of Her2/*neu* Expression in Paraffin-Embedded Breast Carcinomas and Adjacent Normal-Appearing Breast Epithelium

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The combination of *in situ* hybridization and immunohistochemical techniques can successfully identify viral DNA/RNA in specific subsets of cellular populations. We recently modified this method to evaluate amplification of the oncogene Her2/*neu* and overexpression of its protein c-erbB-2 in a series of 15 breast carcinomas. This combination allows the simultaneous evaluation of the oncogene and its corresponding protein expression in single cells and specific cellular populations in histologic tissue sections. Double staining demonstrated heterogeneity within breast carcinomas. In addition, both nuclear and cytoplasmic signals were often detected in morphologically normal-appearing adjacent breast epithelium. The ability to view both the oncogene and its corresponding protein in single cells offers a unique look at the biology of c-erbB-2.

KEY WORDS: Breast cancer, c-erbB-2, Double staining, Her2/*neu*, Immunohistochemistry, *In situ* hybridization.

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Overexpression of the Her2/*neu* oncogene's protein product c-erbB-2 as an indication of poor prognosis in women with breast carcinoma was first described in 1987 by Slamon *et al.* (1). During the next 10 years, numerous investigators found protein overexpression in approximately 25 to 30% of breast cancers. An increased incidence is seen in both intraductal carcinomas, especially of the comedo type, and in Paget's disease of the breast, thus generating the hypothesis that amplification/overexpression of c-erbB-2 is an early event (2–7). The role of this protein, however, which is homologous to the epidermal growth factor receptor, in the etiology and pathogenesis of breast carcinoma remains obscure.

Historically, the evaluation of tissue for protein and gene expression involved serial sections and employed the classic techniques of Southern, Northern, and Western blotting. Problems with sample dilution and sample size often limited the usefulness of these approaches. Slamon *et al.* (8), in a comparative study, reported that immunohistochemical analysis of the Her-2/*neu* in frozen sections offered the best correlation with all other analytic data. After these earlier studies, antibodies effective in paraffin-embedded tissues (9, 10) were developed, eliminating the requirement for frozen samples. In addition, fluorescent *in situ* hybridization (FISH), which allowed evaluation of specific tumor genetics also using paraffin-embedded tissues, has been refined.

The combination of ISH and immunohistochemical techniques has been successful in characterizing viral infections in specific cellular populations (11–13). We describe here a technique combining conventional automated protocols for ISH of the amplified oncogene Her2/*neu* and immunohistochemical techniques for its protein product c-erbB-2 in formalin-fixed, paraffin-embedded breast carcinomas. The combination of these two methods enhances the evaluation of tumor genetics at both the gene and protein level, thus allowing the

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study of oncogene induction and expression in tumor and other related cell populations.

MATERIALS AND METHODS

Case Selection and Scoring

We selected 12 consecutive cases of breast carcinoma from April 1995 through August 1995 that had been routinely immunostained for c-erbB-2 (CB11; BioGenex, San Ramon, CA) and 3 additional cases from 1992 and 1993 under consideration for inclusion in an immune therapy protocol using c-erbB-2 as the target antigen. All of the tissues were fixed in 10% buffered formalin (Biochemical Science, Swedesboro, NJ) and embedded in paraffin, per our laboratory routine. Standard fixation times ranged from 12 to 56 hours. Sections were cut at 4 μm and mounted on ChemMate Capillary Gap Plus slides (Ventana BioTek, Tucson, AZ). Immunohistochemical results from all of the cases was reviewed by the authors and scored as either positive or negative for c-erbB-2. A *negative score* (for immunohistochemical results) meant no staining, weak membrane staining, or weak cytoplasmic staining. A *positive score* meant that the tumors displayed typical strong membrane staining in at least 70% of the tumor cells. The tumors were also scored independently by ISH for amplification of Her2/*neu*. Tumors were considered positive by ISH if a discrete nuclear signal was identified in greater then 70% of the tumor cells.

FISH

FISH using Her2/*neu*, a single-stranded cosmid probe, (Oncor, Gaithersburg, MD) was performed per protocol for tissue ISH from Oncor. In brief, protein digestion was done at 37° C with Oncor protein digestion enzyme, 400 μL in 40 mL of 2× standard saline citrate (SSC) (Proteinase K, 25 mg/mL) for 20 minutes. Slides were then washed, dehydrated, and denatured in a 90° C oven for 12 minutes. Hybridization was performed overnight (16 hr) in a 37° C incubator after slides were placed in a humid box. Post-hybridization washes with agitation were performed, after removal of the coverslip, in 50% formamide/2× SSC (pH 7) at 42° C for 15 minutes. This was followed by a second wash in 0.1× SSC at 37° C for 30 to 35 minutes. The biotinylated probe was visualized with streptavidin-labeled fluorescein after amplification with anti-avidin–labeled fluorescein. Fluorescent slides were viewed with a Zeiss (Thornwood, NY) Axiophot fluorescent microscope using a triple dichroic (4′,6-diamidine-2-phenylindole, fluorescein isothiocyanate, Texas Red) filter (Chroma Technology, Brattleboro, VT).

Double Staining

ISH was performed according to the program established for the BioTek 1000 using BioTek solutions (water and phosphate buffers). Slides were placed in Buffer 2 (BioTek) three times, for 10 minutes each. Protein digestion was carried out with a prediluted Proteinase K enzyme solution (DAKO, Carpinteria, CA) at room temperature for 10 minutes Slides were then washed in Buffer 2 (BioTek) three times, for 10 minutes each. This was followed by three washes in 100% alcohol, 10 seconds each. Slides were then removed from the machine for addition of the probe.

Slides were air dried until the tissue was opacified. Her-2/*neu* probe, prediluted 1:5 in Hybrisol VII (Oncor), was applied according to the manufacturer's directions, approximately 10 μL per 25 × 25-mm surface area. Slides were coverslipped and sealed with rubber cement. After applying the rubber cement, the slides were incubated at 37° C until the rubber cement was clear. The slides were then denatured at 98° C on a hot plate for 13 minutes, and transferred to a 42° C hot plate for 2 hours.

Slides were removed from the hot plate and placed in a Coplin jar filled with low-wash solution, 2× SSC plus 0.2% Tween 20, for removal of the coverslip. Slides were then reloaded in the Techmate oven slide holder, again with care to avoid air bubbles, and placed back on the BioTek 1000. Four low-stringency washes, 2× SSC plus 0.2% Tween 20, at 10 seconds each were followed by three high-stringency washes, 0.1× SSC and 0.2% Tween 20, at 5 minutes each. The high-stringency washes were followed by two Buffer 2 (BioTek) washes, 10 seconds each. Slides were then placed in streptavidin/alkaline phosphatase for 25 minutes. Slides were washed in two Buffer 2 (BioTek) washes, 10 seconds each, and then four Buffer 3 (BioTek) washes, also 10 seconds each. Slides were then stained with three changes of BT Red chromogen (BioTek) for 7 minutes each. Slides were washed in distilled water.

Immunohistochemical Examination

Slides were removed after staining with chromogen and placed in a plastic Tissue Tek Coplin jar filled with citrate buffer (citrate acid monohydrate 10 mM adjusted to pH 6.0 with 2 N sodium hydroxide). Slides were microwaved (Model NN-5602; Panasonic, Danville, KY) on the high setting for 10 minutes, with care used to avoid evaporation during microwaving. After microwave heating, slides were allowed to cool for 20 minutes. They were then rinsed in several changes of distilled water to remove citrate. After rinsing, slides were reloaded on the BioTek 1000.

Immunostaining was performed according to the protocol adapted at Dartmouth-Hitchcock Medical

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Center for the BioTek 1000. Slides were placed in Buffer 2 (BioTek) for 10 seconds, followed by three changes of Buffer 1 (BioTek), 10 seconds each. Primary antibody was then applied (c-erbB-2 clone CB11 (BioGenex) diluted 1:50) for 2 hours at 40° C. Primary antibody application was followed by five Buffer 1 (BioTek) washes, 10 seconds each, then three washes in Buffer 2 (BioTek), 10 seconds each. Secondary antibody was applied for 25 minutes at 40° C. After the application of the secondary antibody, the slides were washed two times in Buffer 2 (BioTek) for 10 seconds each. The avidin-biotin complex (ABC) was applied for 25 minutes at 40° C. After application of the ABC, the slides were washed in Buffer 2 (BioTek) two times for 10 seconds each, followed by three Buffer 3 (BioTek) washes, 10 seconds each. 3,3′-diaminobenzidine was then applied for 5 minutes, followed by two washes in distilled water for 10 seconds each. The slides were then counterstained with hematoxylin (BioTek) for 1 minute. Slides were removed from the BioTek 1000, dehydrated in a series of 95% ethanol followed by three changes of absolute ethanol, and then cleared in four xylene changes. Slides were then coverslipped with Richard Allen mounting medium (Richard Allen Medical, Richland, MI).

RESULTS

Of the 15 breast carcinomas studied, 10 were infiltrating ductal carcinoma, 4 were pure intraductal carcinomas, and 1 was a multifocal papillary carcinoma. Of the infiltrating ductal carcinoma cases, six also had an intraductal component. Overall, 4 (27%) of the 15 tumors were scored as positive for c-erbB-2 overexpression/amplification: 2 infiltrating ductal carcinomas with an associated comedo-pattern intraductal component, 1 multifocal infiltrating ductal carcinoma without an intraductal component, and 1 pure intraductal carcinoma with apocrine features.

Nuclear staining of the Her2/*neu* oncogene was concordant in tumor populations between FISH and the alkaline phosphatase detection used in the double-staining method (Fig. 1). Double staining demonstrated specific nuclear signal only in cells that also showed positive immunostaining for the c-erbB-2 protein (Fig. 1C). Immunohistochemical techniques alone revealed membranous staining in populations similar to those viewed in double-stained sections.

Tumor populations were heterogeneous. Amplification of the gene, although detected in the majority of cells in tumors positive for overexpression of the c-erbB-2 protein, was conspicuously absent in scattered tumor cells with detectable membrane staining (Fig. 2B). In some tumors with very weak

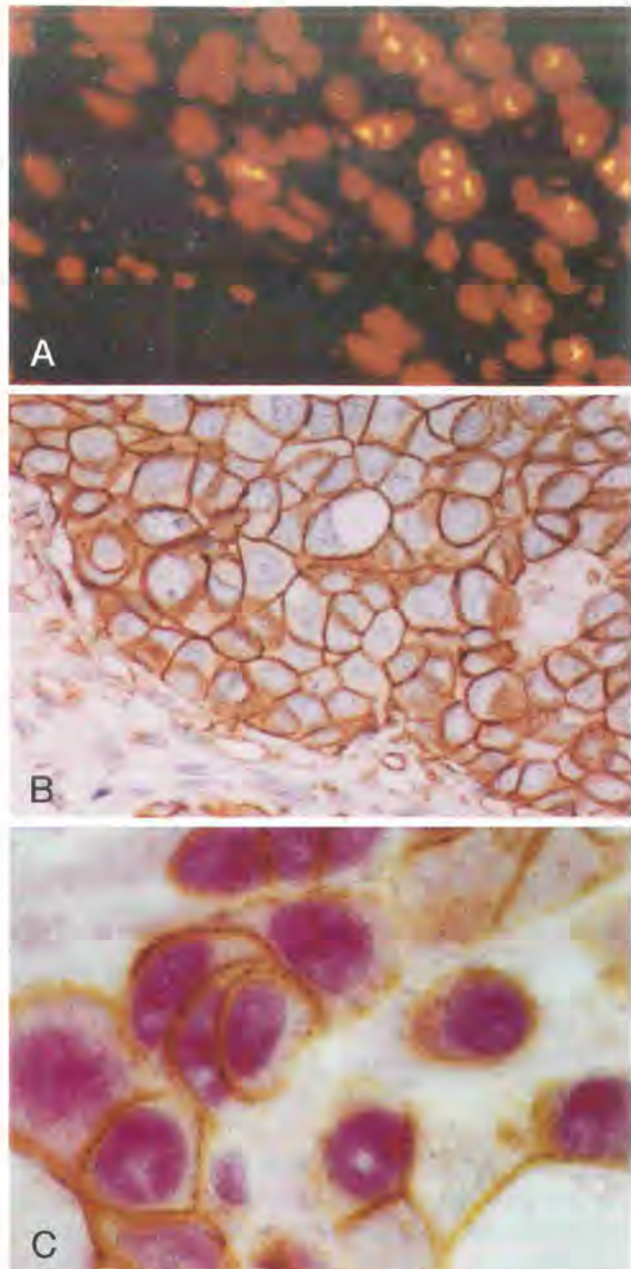

FIGURE 1. Infiltrating ductal carcinoma of breast, highly amplified for the Her2/*neu* oncogene. **A,** FISH for the Her2/*neu* oncogene (propidium iodide counterstain; original magnification, 200×). **B** demonstrates typical strong membrane staining by immunohistochemical analysis alone (original magnification, 200×). **C,** double staining for the Her2/*neu* oncogene (***pink nucleus***) and its protein product c-erbB-2 (***brown membrane***) (ISH with alkaline phosphatase detection combined with avidin-biotin immunoperoxidase and hematoxylin counterstain; original magnification, 300×).

membrane immunostaining for the c-erbB-2 protein (Fig. 3A), double staining exhibited nuclear staining in only a few scattered cells (Fig. 3B). This pattern of weak staining was also observed in adjacent histologically normal-appearing breast epithelium (Fig. 4). The converse, gene amplification without protein expression, was not identified in this study.

As predicted, the major factor in determining quality of staining was tissue fixation. In tissues less

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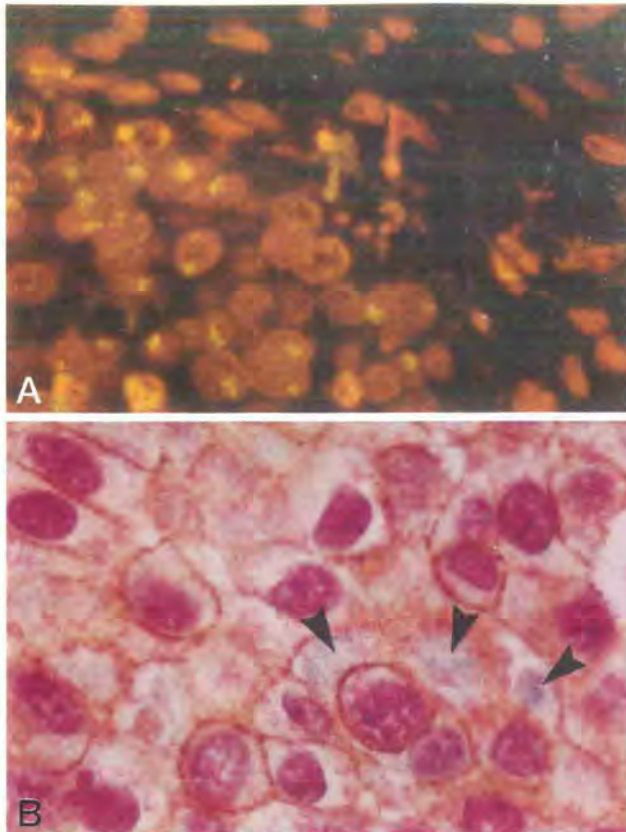

FIGURE 2. Infiltrating ductal carcinoma of breast with less amplification of the Her2/*neu* oncogene. **A,** FISH for the Her2/*neu* oncogene (propidium iodide counterstain; original magnification, 200×). **B,** double staining for the Her2/*neu* oncogene (***pink nucleus***) and its protein product c-erbB-2 (***brown membrane***). Note several tumor cells with membrane staining and no nuclear signal (***arrowheads***) (ISH with alkaline phosphatase detection combined with avidin-biotin immunoperoxidase and hematoxylin counterstain; original magnification, 250×).

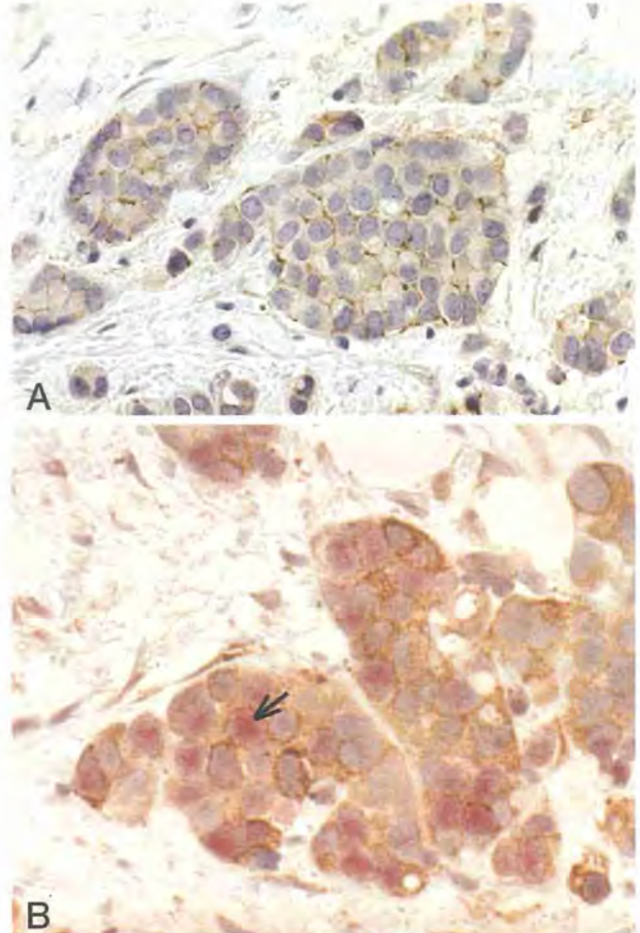

FIGURE 3. Infiltrating ductal carcinoma, male breast. **A** demonstrates weak membrane staining by immunohistochemical analysis alone (original magnification, 150×). **B,** double staining showing nuclear signal (***arrow***) in scattered cells (ISH with alkaline phosphatase detection combined with avidin-biotin immunoperoxidase and hematoxylin counterstain; original magnification of all panels, 200×).

well fixed, greater than one-half of the protein signal was lost after increasing the time of Proteinase K digestion. Conversely, decreasing protein digestion by decreasing the time of digestion resulted in loss of ISH. Additional dilution of the prediluted Proteinase K, 1:3 and 1:5, resulted in preservation of the antigen but loss of hybridization signal. Although time and concentration must be determined empirically, our best results to date far have been obtained with the DAKO prediluted Proteinase K used with no additional dilution at room temperature, followed by antigen retrieval. Generally, the double-stained slides contained more cytoplasmic signal than the same tissue stained by immunohistochemical techniques alone. This pattern was most likely related to the Proteinase K digestion step required for ISH. The CB11 antibody recognizes an intracytoplasmic component of the membrane-bound c-erbB-2 protein, and cleavage during digestion could easily result in a less crisp membrane stain.

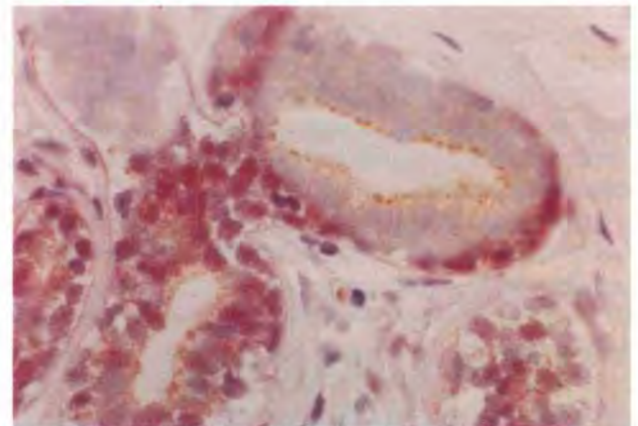

FIGURE 4. Normal-appearing breast lobule with weak membrane and cytoplasmic staining for c-erbB-2 (***brown***) but only scattered nuclei positive for the Her2/*neu* oncogene (***pink***) (ISH with alkaline phosphatase detection combined with avidin-biotin immunoperoxidase and hematoxylin counterstain; original magnification, 200×).

CONCLUSIONS

Detection of Her2/*neu*/c-erbB-2 by alkaline phosphatase ISH coupled with ABC 3,3′-diamino-

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benzidine immunohistochemical analysis in tumor populations correlated with single staining by either immunohistochemical techniques alone or FISH alone. Heterogeneity for gene amplification within the tumor population was demonstrated by double staining as a lack of nuclear signal in individual tumor cells that demonstrated clear membrane immunostaining. This phenomenon was found within tumors that had strong membrane staining revealed by immunohistochemical methods as well as in tumors with less staining. In populations with faint membrane staining, however, there were fewer cells with nuclear signal than there were in the populations with strong membrane staining. Heterogeneity for gene amplification within tumor populations has been described using FISH, but intratumoral heterogeneity is often not recognized by immunohistochemical analysis alone (14, 15). In this study, immunohistochemical techniques by themselves did not equate to the presence of gene amplification as detected by ISH for an individual tumor cell.

Tumor populations that overexpress the c-erbB-2 protein without gene amplification have been described in approximately 3 to 10% of breast carcinomas studied by a combination of methods, *i.e.*, FISH, immunohistochemical techniques, and Southern blot analysis (1, 8, 14, 16). Hollywood and Hurst (17) demonstrated a novel DNA binding protein, OB2–1, in cell lines overexpressing the c-erbB-2 protein and its corresponding mRNA but containing a single gene copy. This DNA-binding protein was increased in amplified tumors as well. OB2–1 was not increased in populations of cells with weak membrane staining. These observations led some authors to conclude that it is the overexpression of the membrane protein detected by immunohistochemical means that best predicts tumor behavior (4). In support of this opinion, tumor cells that had Her2/*neu* gene amplification but no protein expression were not identified in this study.

The appearance of nuclear signal in scattered epithelial cells in the adjacent normal-appearing breast epithelium that showed weaker membrane staining by the CB11 antibody was even more interesting. Weak membrane staining for c-erbB-2 protein in normal breast epithelium has been previously described, and this level of staining is thought to correspond to the level identified in nonamplified breast cancers that contain a single copy of the gene by Southern blot analysis (18, 19). Identification of a few morphologically benign cells with nuclear signal might indicate an early increase in gene copy number, which could herald neoplastic transformation. This observation is concordant with that of Kallioniemi *et al.* (15), who found no extrachromosomal *erb*B-2 genes in amplified populations. They suggested that if extrachromosomal amplification occurred, it would have happened in a preclinical phase of tumor growth. Accumulation of genetic abnormalities as loss of heterozygosity in morphologically normal-appearing breast tissue was recently described (20), and our results provide additional evidence of genetic changes in histologically normal-appearing breast epithelium adjacent to tumor.

Methodologic problems encountered were not unique to double staining. Fundamentally, the quality of the tissue fixation was the key to good results. The biggest drawback to performing this procedure using an automated stainer was the amount of probe required for coverage with the capillary gap action. The cost of the commercially available probe made it prohibitive to use the stainer during hybridization. Slides were removed from the stainer just before denaturation, to conserve probe, and the procedure continued manually. The slides were returned to the machine after hybridization for the rest of the procedure.

After double staining, more cytoplasmic signal was present in some of the tissues than was found in the same tissue stained with immunohistochemical methods alone, which gave only membrane staining. A likely explanation for this additional cytoplasmic staining is that the Proteinase K digestion is cleaving some of the membrane-bound protein, which is then detected in the cytoplasm, because the CB11 antibody is targeted to an intracytoplasmic portion of this molecule. The level of membrane staining correlated with the intensity of nuclear staining in the majority of the tumor cells, thus reconfirming a relationship between gene amplification and membrane protein expression.

In conclusion, the ability to study both the phenotype and genotypic expression of c-erbB-2 in single cells within various tumor and nontumorous populations offers a unique view of its expression in the breast. The majority, approximately 70%, of the carcinomas that we studied were considered negative for overexpression of the oncogene, which is consistent with data reported by other investigators. Nevertheless, as demonstrated in Figure 3, most of the tumors we studied did show some staining with the CB11 antibody and scattered positive nuclei by ISH. Our data suggest that amplification of the gene is an early event that might in some cases precede overexpression of the protein, because we did not see hybridization in scattered individual cells in both nontumor and tumor populations that showed weak expression of the protein. These findings are an additional confirmation of the theory that breast carcinoma, much like carcinoma in the colon, results from a multistage process, with the progressive accumulation of genetic alterations along the way.

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