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ERBB2 amplification in breast cancer analyzed by fluorescence *in situ* hybridization

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ABSTRACT **We illustrate the use of fluorescence *in situ* hybridization (FISH) for analysis of *ERBB2* oncogene copy number, the level of amplification (here defined as the ratio of *ERBB2* copy number to copy number of chromosome 17 centromeres), and the distribution of amplified genes in breast cancer cell lines and uncultured primary breast carcinomas. The relative *ERBB2* copy number determined by FISH in 10 breast cancer cell lines correlated strongly with Southern blot results ($r = 0.98$) when probes for an identical reference locus were used in the two methods. Metaphase analysis of cell lines showed that amplified *ERBB2* copies always occurred in intrachromosomal clusters but that the number and chromosomal location of these clusters varied among the cell lines. In interphase nuclei of primary tumors showing *ERBB2* amplification (10/44), *ERBB2* copies were seen as one to four clusters, also suggesting intrachromosomal localization. Regardless of the average level of amplification, all these tumors contained highly amplified cell subpopulations with at least 25, and sometimes more than 100, *ERBB2* copies per cell. Tumors that did not show amplification by FISH (34/44) had an average of one to five *ERBB2* copies scattered randomly in the nuclei and completely lacked cells with high copy levels. FISH results on primary tumors were concordant with slot blot results on amplification and with immunohistochemical detection of overexpression. Quantitative analysis of *ERBB2* amplification by FISH may improve prognostic assessments based on the pattern of amplification and detection of heavily amplified tumor cell subpopulations.**

Gene amplification is a characteristic feature of cancer cells that allows increased production of specific proteins needed for acquisition and maintenance of the malignant phenotype (1–6). Amplification of certain oncogenes has an important role in the progression of many tumors (3–5). For example, *MYCN* oncogene is amplified in neuroblastomas, *MYC* and *MYCL* are amplified in small cell lung cancer, and *ERBB2* is amplified in breast and ovarian cancer. Detection of such amplifications may, in some instances, assist in diagnosis and in prognostic assessment. Gene amplification also contributes to the generation of resistance to cytotoxic drugs.

Analyses of established cancer cell lines indicate that amplified oncogenes may be present either in extrachromosomal double-minute chromosomes or in expanded chromosomal regions, also called homogeneously staining regions (3, 4). Cytogenetic studies of short-term cell cultures from malignant human tumors indicate that double-minute chromosomes are more common than expanded chromosomal regions (6). Episomes, small circular DNA molecules that may be precursors of double-minute chromosomes, have been shown to carry amplified *MYCN* oncogenes in uncultured neuroblastomas (7). Conventional Southern analyses of primary tumors provide no information on these patterns of amplification. Southern blot assays also cannot evaluate the characteristic intratumor heterogeneity that may contribute to the genetic progression of the neoplasia (8). Thus, more informative procedures for analysis of gene amplification in primary tumors are needed.

ERBB2 (*Her-2/neu*) oncogene, which codes for a 185-kDa transmembrane growth factor receptor, is amplified (9–16) and/or overexpressed (17–23) in 15–25% of breast carcinomas. Association of *ERBB2* amplification and overexpression with rapid proliferation (15, 22), low estrogen receptor content (9, 15, 19, 21, 22), and high grade (16, 20–22) of ductal carcinomas suggests that this oncogene plays an important role in the progression of breast cancer. Many studies have shown that amplification or overexpression of *ERBB2* is an indicator of poor prognosis in breast cancer (9, 12, 13, 15, 16, 19, 20–22). However, some studies have failed to find a significant association of *ERBB2* with survival (11, 14, 18). The disparate findings may be in part due to technical difficulties in accurately assessing the level of gene amplification (11, 13) or of protein expression (13, 22, 23). Previous studies have provided no information on the molecular mechanisms of *ERBB2* amplification *in vivo*.

We show here that fluorescence *in situ* hybridization (FISH) allows assessment of the level of *ERBB2* amplification as well as the spatial distribution of oncogene copies in individual uncultured primary breast carcinomas. The method is based on two-color FISH, where hybridization of a cosmid contig probe for the *ERBB2* gene is detected with fluorescein isothiocyanate (FITC), and hybridization of a probe to chromosome 17 pericentromeric sequences (or another reference locus) is detected with Texas Red.

MATERIALS AND METHODS

Cells and Cell Preparation. Ten established breast cancer cell lines (BT-474, SK-BR-3, MDA-361, MDA-453, MDA-468, MCF-7, BT-549, BT-20, BT-483, and Hs578T) and one normal human skin fibroblast cell line were obtained from the ATCC. The cell lines were either treated with Colcemid (0.05 μg/ml) for 2–4 hr to obtain metaphase preparations or harvested at confluency to obtain G_1 phase enriched interphase nuclei. Primary breast cancer samples were obtained by extensive fine needle aspiration of the surgical tumor specimens, by mechanical disaggregation of fresh tumor tissues, or by touching a freshly cut tumor surface to a microscope slide (touch preparations). All samples were fixed in methanol/acetic acid (3:1). The histological diagnoses of the 44 primary tumors were ductal invasive (42 cases), lobular invasive (1 case), and intraductal carcinoma (1 case). Twenty-two (50%) of the tumors were from node-positive patients.

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Abbreviations: FISH, fluorescence *in situ* hybridization; DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

DNA Probes and Probe Labeling. Two contiguous *ERBB2* cosmid clones (cRCNeu1 and cRCNeu4), together spanning 55 kilobases of genomic DNA, were obtained from Richard M. Cawthon, Peter O'Connel, and Ray White (Howard Hughes Medical Institute, Salt Lake City). Cosmid DNA was labeled with digoxigenin-11-dUTP by nick-translation. These probes gave a specific hybridization signal in lymphocyte metaphase chromosomes at 17q12–21.1. Three other probes (labeled with biotin-14-dATP) were used in dual-color hybridizations: a cK17.10 cosmid probe (from A.K.) hybridizing to the pericentromeric repeat sequence on chromosome 17, a D21S16 cosmid probe (from S. Rijder and D. Cox, University of California, San Francisco), and a whole chromosome probe for chromosome 17 (DNA from a chromosome 17 cosmid library).

***In Situ* Hybridization.** A dual-color FISH was performed with digoxigenin-labeled *ERBB2* probes and biotin-labeled reference probes (24, 25). All samples were treated with 1 μg per 50 ml of proteinase K (Boehringer Mannheim) for 7.5 min at 37°C before hybridization. Cells on slides were denatured in 70% formamide/2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 70°C for 2–3 min and dehydrated in ethanol. Ten microliters of hybridization mixture, consisting of a total of 40 ng of labeled probes, 0.5 μg of unlabeled, sonicated (200–500 base pairs) human placental DNA in 50% formamide/10% dextran sulfate/2× SSC, was denaturated for 5 min at 70°C, allowed to reanneal for 45–60 min at 37°C, and applied to denatured cells on slides. Hybridization was done overnight at 37°C under a coverslip in a moist chamber.

Immunofluorescent Probe Detection. The slides were washed three times in 50% formamide/2× SSC, twice in 2× SSC, and once in 0.1× SSC for 10 min each at 45°C. After washing, the slides were immunocytochemically stained at room temperature in three steps (30–45 min each). Before the first immunocytochemical staining, the slides were preblocked in 1% bovine serum albumin (BSA)/4× SSC for 5 min. The first staining step consisted of 2 μg of Texas Red avidin per ml (Vector Laboratories) in 1% BSA/4× SSC. The slides were then washed in 4× SSC, 4× SSC/0.1% Triton X-100, 4× SSC, and PN [a mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 (pH 8), and 0.1% Nonidet P-40] for 10 min each and preblocked with PNM (5% Carnation dry milk/0.02% sodium azide in PN buffer) for 5 min. The second antibody incubation consisted of 20 μg of FITC-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) and 5 μg of anti-avidin antibody (Vector Laboratories) per ml in PN buffer followed by three PN washes (10 min each). After PNM block, the third immunochemical staining was done with rabbit anti-sheep FITC antibody (1:50 dilution) (Vector Laboratories) and 2 μg of Texas Red avidin per ml in PN. After three PN washes, nuclei were counterstained with 0.2 μM 4',6-diamidino-2-phenylindole (DAPI) in an antifade solution.

Fluorescence Microscopy and Scoring Criteria. A Nikon fluorescence microscope equipped with a double band-pass filter (Chroma Technology, Brattleboro, VT) and a ×100 objective was used for simultaneous visualization of FITC and Texas Red signals. At least 150 nuclei with intact morphology on the basis of DAPI counterstaining were scored from each clinical specimen. Clumps, overlapping nuclei, and tumor infiltrating leukocytes were ignored. Only nuclei with unambiguous chromosome 17 centromeric hybridization signals were scored for the *ERBB2* signal number. In cases of high levels of gene amplification, 50 cells were scored in more detail to determine the gene copy number and the pattern of gene amplification. The scoring results from primary tumors were expressed both as an absolute *ERBB2* copy number per cell and as the level of amplification (*ERBB2* copy number relative to the chromosome 17 centromere copy number).

Southern and Slot Blot Analysis. High molecular weight DNA isolated from breast cancer cell lines (26) was digested with *Bam*HI, fractionated by agarose gel electrophoresis, and transferred to Hybond N+ membranes (Amersham). The membranes were hybridized with ^{32}P-labeled DNA probes for *ERBB2* (pMAC117; ATCC) and D21S16 (a plasmid probe for D21S16 matching the cosmid probe used in FISH) and exposed to Kodak X-Omat XAR-5 film for 1–4 days at −70°C. The hybridization signals were quantitated with a densitometer. The level of amplification was calculated by dividing the ratio of PMAC117 probe signal to the D21S16 probe signal in the breast cancer cell lines by the average ratio of these signals in normal tissues. The presence of *ERBB2* amplification in primary tumors was determined by slot blot analysis based on the ratio of the pMAC117 signal to the signal produced by ^{32}P-labeled total human genomic DNA (27). A ratio exceeding the values obtained from normal breast tissues by 3 SD was taken to represent amplification.

Immunohistochemical Analysis of *ERBB2* Overexpression. ERBB2 protein was stained as described (28) using an immunoperoxidase technique and an affinity-purified OA-11-854 polyclonal antibody (1:2500 dilution; Cambridge Research Biochemicals, Valley Stream, NY) specific for the internal domain of the ERBB2 protein. Samples were scored for percentage immunopositive cells and were considered positive if >25% of the cancer cells showed membranous staining.

RESULTS

Two green *ERBB2* signals (and two red chromosome 17 pericentromeric probe signals) were observed in ≈90% of G_1 phase enriched interphase nuclei of normal fibroblasts (Fig. 1*a*). The rest of the cells had either one (5%), three (3%), or four (2%) *ERBB2* signals. In G_2 phase enriched lymphocytes, 12% and 30% of nuclei showed three and four signals, respectively.

FISH was applied to analysis of the level of *ERBB2* amplification (relative to chromosome 17 centromere probe) and actual gene copy number in interphase nuclei of 10 established breast cancer cell lines (Table 1). MDA-361 and MDA-453 cells had 2.5- to 3-fold amplification (≈11 *ERBB2* signals per cell) and SK-BR-3 and BT-474 cells had 8- to 11-fold amplification (≈45 signals per cell) (Fig. 1*b*). The other cell lines had no gene amplification, although they still contained an average of 2.5–3.5 *ERBB2* signals per cell, apparently because of aneuploidy and the presence of S- and G_2/M-phase cells. In all cell lines, the actual *ERBB2* copy levels per cell varied within a 2-fold range from G_1 phase to G_2/M phase.

Our FISH results were concordant with previous Southern blot estimates of *ERBB2* amplification in these cell lines (29, 30) (Table 1). However, as the loci used as references in these studies (*mos* oncogene or β-actin genes in Southern blots and chromosome 17 centromere in FISH) were different, we made additional measurements to compare Southern and FISH analyses of relative copy number using probes for an identical reference locus (D21S16) in both methods (Table 1). FISH and Southern blot results on *ERBB2* amplification relative to the D21S16 locus were very closely correlated ($r = 0.98$).

Analysis of metaphase preparations from the four breast cancer cell lines with *ERBB2* amplification indicated that amplified genes were always integrated in chromosomes (Fig. 1*c*). In the highly amplified cell lines (SK-BR-3 and BT-474), clusters of *ERBB2* signals were seen on numerous chromosomes. The chromosomal distribution of *ERBB2* signals was different among the four cell lines with most of the clusters occurring on chromosomes other than 17 (Table 2). The number of *ERBB2* copies in a typical cluster varied from 6 to

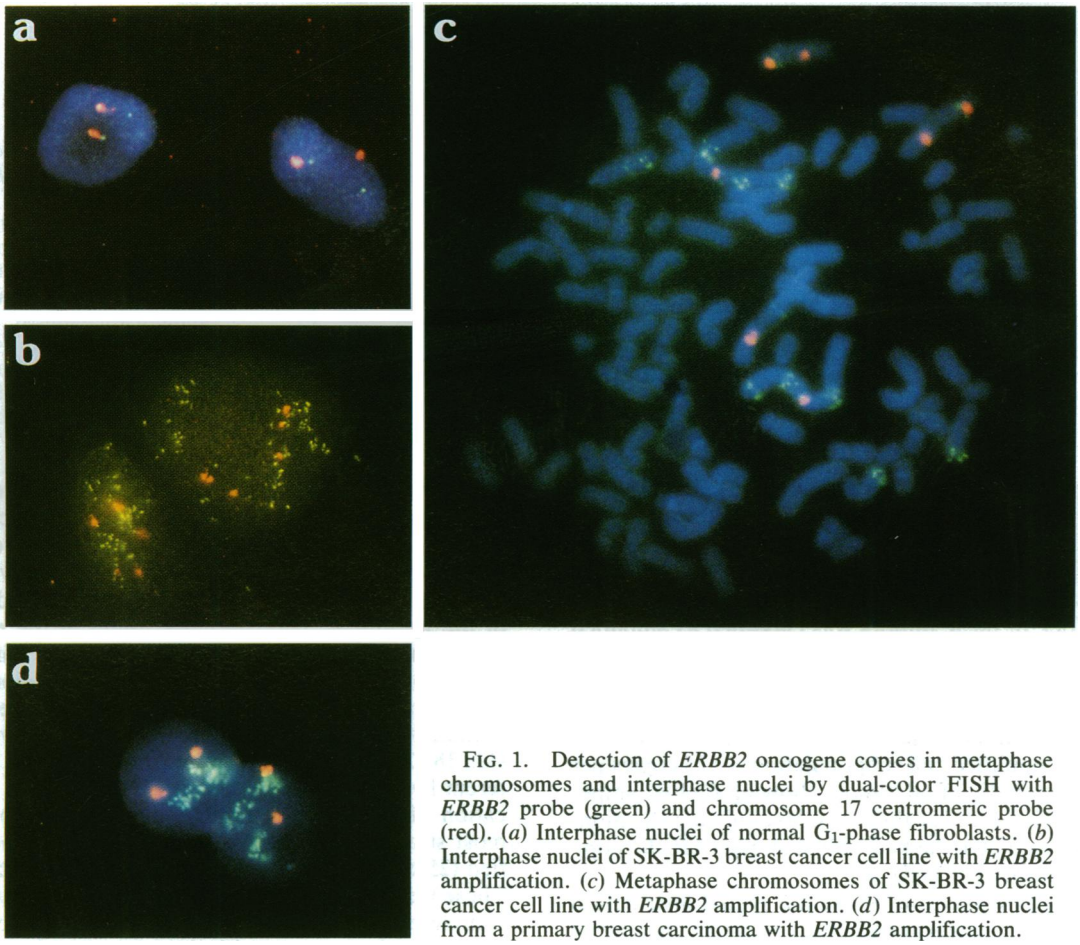

FIG. 1. Detection of *ERBB2* oncogene copies in metaphase chromosomes and interphase nuclei by dual-color FISH with *ERBB2* probe (green) and chromosome 17 centromeric probe (red). (*a*) Interphase nuclei of normal G_1-phase fibroblasts. (*b*) Interphase nuclei of SK-BR-3 breast cancer cell line with *ERBB2* amplification. (*c*) Metaphase chromosomes of SK-BR-3 breast cancer cell line with *ERBB2* amplification. (*d*) Interphase nuclei from a primary breast carcinoma with *ERBB2* amplification.

20 and the number of clusters per cell line varied from 1 to 10. The complex karyotype of these cells made the detailed determination of the sites of amplification difficult. For example, the SK-BR-3 cell line contained two long marker chromosomes with three *ERBB2* clusters. Although these chromosomes reacted with a chromosome 17 centromeric probe (Fig. 1*c*), they did not stain at all with the respective whole chromosome probe. In addition to *ERBB2* clusters, the BT-474 cell line also contained single integrated *ERBB2* copies in chromosomes other than 17.

FISH also was applied to determine the actual *ERBB2* copies per cell (Fig. 2*A*) and the level of amplification (defined here as *ERBB2* copy number relative to the chromosome 17 centromere copy number) in interphase nuclei of 44 primary breast carcinomas (Fig. 2*B*). Ten tumors (23%) showed >2-fold amplification by FISH with an average of 8–55 *ERBB2* signals per cell arranged in one to four clusters in the interphase nuclei (Fig. 1*d*). All 10 tumors showed overexpression of *ERBB2* by immunohistochemistry and 8 of them also were amplified by slot blot analysis. Two tumors not classified as amplified by slot blot analysis showed 6- to 7-fold amplification (17 or 18 *ERBB2* copies per cell) by FISH and overexpression of *ERBB2* by immunohistochemistry. One of these tumors had a prominent leukocyte infiltration

Table 1. Comparison of absolute and relative *ERBB2* copy numbers detected by FISH with Southern analysis in 10 breast cancer cell lines

	FISH analysis, mean copy no.			Southern analysis, level of amplification	
Cell line	*ERBB2* per cell	*ERBB2* per 17cen	*ERBB2* per D21S16	*ERBB2* per mos/act	*ERBB2* per D21S16
BT-474	47	11	13.5	4–8	12.5
SK-BR-3	43	8	9	4–10	7.5
MDA-361	11	3	4	2–4	6
MDA-453	11	2.5	3	2	2.5
MDA-468	2.5	1	1	1	1
MCF-7	2.5	1	1	<1	1
BT-549	2.5	1	1	1	NT
BT-20	3	1	1	1	NT
BT-483	3.5	1	1	<1	NT
Hs578T	3	1	1	1	NT

NT, not tested; 17cen, chromosome 17 centromere probe signals; D21S16, signals from a D21S16 cosmid probe; mos/act, *mos* oncogene or β-actin (28, 29).

Table 2. Distribution of *ERBB2* signals on chromosome 17 and on other chromosomes in four breast cancer cell lines

	No. of *ERBB2* clusters		No. of single *ERBB2* signals	
Cell line	On Chr 17	On other Chr	On Chr 17	On other Chr
SK-BR-3	0	7 or 8	3	1
BT-474	2	7 or 8	3 or 4	2 or 3
MDA-361	0	1	4	0
MDA-453	2	0	2	0

Identification of chromosomes with *ERBB2* copies was based on DAPI-stained metaphase preparations and simultaneous dual-color FISH with *ERBB2* and chromosome 17 centromere or whole chromosome probes. Chr, chromosome(s).

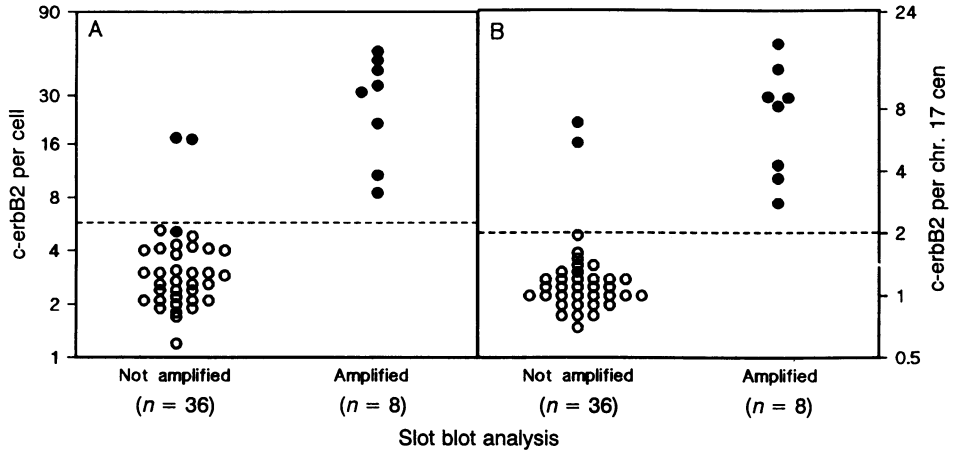

FIG. 2. Comparison of FISH results on mean *ERBB2* copy numbers per cell (*A*) and mean *ERBB2* copy numbers per chromosome 17 centromere signal number (*B*), with slot blot analysis of *ERBB2* amplification and immunohistochemical analysis of overexpression in 44 primary breast carcinomas. Tumors showing positive (●) and negative (○) staining by immunohistochemistry are indicated.

that may have interfered with the slot blot analysis. The remaining 34 tumors did not appear to be amplified by FISH. They did not show *ERBB2* clusters and had on average one to five *ERBB2* signals per cell and an equal number of chromosome 17 centromeric signals distributed at random in the nuclei. None of these tumors was classified as amplified by slot blot analysis and only one showed overexpression by immunohistochemistry.

The 10 primary tumors with *ERBB2* amplification by FISH showed a high intratumor heterogeneity (Fig. 3). The presence of heavily amplified cell subpopulations (>25 copies per cell) was characteristic of all tumors showing *ERBB2* amplification by FISH, whatever the mean level of amplification. Other tumors completely lacked cells with high *ERBB2* copy levels (>8–12 copies per cell).

FISH made it possible to evaluate the spatial distribution of amplified *ERBB2* signals in interphase nuclei of uncultured primary breast carcinomas. The amplification was always seen as clusters of *ERBB2* signals (Fig. 1*d*), suggesting intrachromosomal amplification. The number of clusters per cell varied (from one to four) as did the number of *ERBB2* copies per cluster (from 4 to ≈25) both between and within the tumors.

DISCUSSION

The major advantage of FISH as compared with other methods for quantitating *ERBB2* gene amplification is that it allows measurement of the average level of amplification in a tumor as well as the actual number and distribution of *ERBB2* genes in individual, morphologically defined cells. The method is rapid, nonradioactive, and requires very little

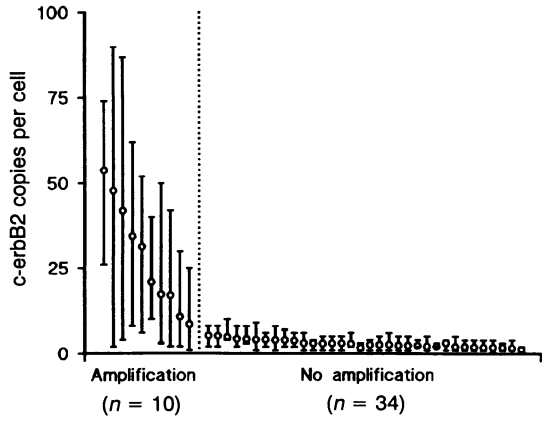

FIG. 3. Mean *ERBB2* copy number and 90% range in individual interphase nuclei of 44 primary breast carcinomas.

tumor material. Our results indicate that the average level of *ERBB2* amplification determined by FISH is closely correlated with Southern and slot blot data. However, in tumors with amplification, FISH revealed an extensive cell to cell variation in gene copy number. This is expected on the basis of the intratumor heterogeneity found in the evaluation of other tumor properties such as histologic differentiation, DNA content, or estrogen receptor expression. However, such intratumor heterogeneity has often not been detected in immunohistochemical analyses of *ERBB2* overexpression (13, 18, 22), probably because the distinction between different levels of overexpression is not possible. The identification of tumors with *ERBB2* amplification by FISH was most straightforward based on the finding of heavily amplified (25 to >100 copies per cell) tumor cell subpopulations. These were present in all tumors with amplification but were completely absent from those without amplification. These results are in contrast with those commonly reported from Southern analyses, which indicate a more or less gradual transition between tumors with and without gene amplification (9, 11, 13, 15). Previous reports based on Southern analysis have indicated that the level of gene amplification is a better prognostic predictor in breast cancer than the mere presence or absence of gene amplification (9, 13, 16). The relation of *ERBB2* amplification with clinical outcome may improve when the occurrence of small subpopulations of heavily amplified cells, the degree of heterogeneity in gene amplification, as well as the different patterns of amplification (size, number, and morphology of *ERBB2* clusters in interphase nuclei) are taken into account.

FISH allows the distinction between elevated *ERBB2* copy number caused by specific gene amplification and that by chromosomal duplication (4, 11). As shown in our study, tumors with an average of three to five *ERBB2* copies due to aneusomy are common. These tumors typically had scattered *ERBB2* signals and an equal number of chromosome 17 centromere signals. Except for one case, no *ERBB2* gene overexpression was found in these cases.

FISH is a potential alternative to immunohistochemistry for assessment of *ERBB2* activation. The two assays were found to be correlated but FISH may be more quantitative. The quantitative nature of immunohistochemical analysis is limited by the physicochemical properties of the immunoperoxidase reaction and the subjective assessment of staining intensity. The detection of overexpression also is dependent on variation in the degree of tissue fixation and the specificity of the antibodies used (13, 22, 23). Western blotting, a potentially more quantitative assay of overexpression, is often confounded by the dilution effect caused by stromal proteins and has been shown to be the most inaccurate method for *ERBB2* analysis (13). Of course, FISH will not detect *ERBB2* activation if it occurs as a result of increased

gene transcription with no change in gene copy number. Previous studies indicate that some breast carcinomas with *ERBB2* overexpression do not have gene amplification (13, 17, 19). We found two tumors with *ERBB2* overexpression, which were classified as amplified by FISH but not by slot blot, suggesting that tumors with overexpression but no gene amplification may be less frequent than previously thought.

A unique property of FISH is that it allows evaluation of the pattern of amplification on the basis of spatial distribution of amplified gene copies in interphase nuclei and in metaphase chromosomes. Metaphase analysis of the breast cancer cell lines indicated that amplified genes were always intrachromosomal but distributed in many different chromosomes. The appearance of amplified *ERBB2* copies as two to four clusters in the interphase nuclei of primary breast carcinomas also suggests intrachromosomal location. *ERBB2* copies carried in extrachromosomal structures such as double minutes and episomes are less likely to be clustered in interphase cells. Cytogenetic studies of primary breast carcinomas have revealed a high prevalence of homogeneously staining regions but no double minutes, suggesting the predominance of intrachromosomal gene amplification (31). The distance between the *ERBB2* hybridization signals in each cluster indicates that the amplicons typically span at least a few hundred kilobases (32).

The pattern of amplification as defined by the distribution of *ERBB2* signals in interphase nuclei may reflect the mechanism of gene amplification. According to the episome model of oncogene amplification, extrachromosomal amplification is an early step, whereas the integration of amplified genes into chromosomes occurs later during tumor progression (6). This model is supported by the frequent cytogenetic findings of double minutes in short-term cultures of various primary tumors as well as by the presence of episomal amplification of *MYCN* in neuroblastomas *in vivo* (6, 7). As we found no evidence of extrachromosomal *ERBB2* genes in breast cancer, it is likely that extrachromosomal amplification, if it existed, must have occurred in the preclinical phase of tumor growth. Gene amplification in cell lines selected by increasing drug concentrations often first appears on a single chromosome carrying the original gene locus (33). There is no evidence of double-minute chromosomes as precursors of intrachromosomal structures. Initially, multiple copies of very large sequences may be amplified on a single chromosome, whereas later more condensed gene clusters are formed that also may translocate to other chromosomes (33, 34). The end result is a pattern similar to that observed here in the highly amplified breast cancer cell lines. Translocations cannot, however, explain the pattern of amplification in some breast cancer cell lines, as *ERBB2* copies also were found in metaphase chromosomes showing no staining with the whole chromosome probe for chromosome 17. The breast cancer cell lines as well as primary tumors studied here have had considerable opportunity to evolve since the initial amplification events. Analysis of the *ERBB2* amplification pattern by FISH in very early breast lesions such as *in situ* carcinomas could further clarify the mechanism of *ERBB2* amplification in breast cancer.

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