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ERBB-2 (*HER2/neu*) Gene Copy Number, p185^{HER-2} Overexpression, and Intratumor Heterogeneity in Human Breast Cancer[1]

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ABSTRACT

Amplification of the *ERBB-2* (*HER-2/neu*) gene is accompanied by overexpression of its cell surface receptor product, p185^{HER-2}. Heterogeneity has been observed for both the gene copy number and the level of overexpression of its protein product. To better understand their relationship, correlation between the level of cellular expression of p185^{HER-2} and *ERBB-2* gene amplification was studied in four human breast cancer cell lines (BT-474, SK-BR-3, MDA-453, and MCF-7) and in a primary human breast tumor sample. The relative expression of p185^{HER-2} was measured by immunofluorescence by using flow and/or image cytometry while correlated DNA analysis was performed on the same cells by fluorescence *in situ* hybridization to determine *ERBB-2* gene and chromosome 17 copy numbers. Marked heterogeneity was observed in both protein expression and *ERBB-2* copy number. Despite this heterogeneity, and in accordance with previous studies, the average levels of p185^{HER-2} expression correlated well with average *ERBB-2* gene copy numbers in the four lines examined ($r = 0.99$). When the relationship between copy number and protein expression was studied on a cell-by-cell basis, p185^{HER-2} expression correlated with both the absolute number of *ERBB-2* gene copies/cell ($r = 0.59–0.63$) and chromosome 17 copy number ($r = 0.45–0.61$). It is of interest that there was weak or no correlation between p185^{HER-2} protein expression and the *ERBB-2* copy number:chromosome 17 copy number ratio ($r = 0.0–0.25$). In more than one-half of cells expressing a high level of p185^{HER-2}, the chromosome 17 copy number was high (two or three times the average copy number), whereas <2% of an unselected population had a high chromosome 17 copy number. Bromodeoxyuridine incorporation indicated that the S-phase-labeling index was homogeneous across various p185^{HER-2}-expressing subpopulations in the SK-BR-3 cell line. Analysis of the primary breast tumor sample showed results similar to the cell lines, supporting the strong possibility of a mechanistic link among p185^{HER-2} overexpression, *ERBB-2* amplification, and high chromosome 17 copy number.

INTRODUCTION

A characteristic feature of cancer cells is the unregulated expression of genes involved in cellular growth control. One of these genes is the *ERBB-2* (*HER-2/neu*) proto-oncogene, which encodes a M_r 185,000 transmembrane glycoprotein (p185^{HER-2}) that belongs to a subfamily of growth factor receptors having intrinsic tyrosine kinase activity, including the epidermal growth factor receptor and the receptors HER-3 and HER-4 (1–3).

Amplification and overexpression of *ERBB-2* is found in 25–30% of primary human breast cancers and is associated with a poor clinical outcome (4–6). This suggests that overexpression of p185^{HER-2} plays a role in the pathogenesis of some human breast cancers (5, 6). Although overexpression of p185^{HER-2} is usually accompanied by amplified *ERBB-2* in tumor DNA, rare breast tumors overexpress p185^{HER-2} protein or c-*ERBB-2* mRNA levels in the absence of detectable gene amplification (7).

Although amplification of *ERBB-2* is generally considered to be a significant prognostic indicator in patients with breast cancer, its applicability continues to be controversial, in part because of analytical discrepancies associated with the methods traditionally used to evaluate its amplification and/or overexpression. These techniques include Southern blotting, slot blot analysis, and FISH[4] for detection of amplification, while ELISA, Western blotting, immunohistochemistry, and immunofluorescence are used to evaluate overexpression (8–15). Because FISH allows the observer to distinguish small subpopulations of amplified cells, it is more sensitive than blotting techniques. In addition, FISH allows one to identify particular locations where aberrations exist in single tumor specimens (10, 14). Similarly, because immunohistochemically stained slides are difficult to quantify and because ELISA and Western blotting data do not provide information concerning heterogeneity, immunofluorescence has advantages over these other methods (13, 14).

Marked heterogeneity has been described in primary breast cancers in both the copy number of *ERBB-2*/cell and in the level of p185^{HER-2} protein (5–12). Although cell-to-cell differences may be due in part to analytical variation, genetic and epigenetic dispersion may also play significant roles. This heterogeneity provides a potential source for the selection of subclones with increased malignant and metastatic potential, especially in the context of therapeutic targeting based on *ERBB-2* expression.

Although amplification of *ERBB-2* correlates well with overexpression of p185^{HER-2} protein in cell populations (5, 6, 9, 11, 14–16), the correlation has not been made on a cell-by-cell basis. The present communication describes our analysis of the extent to which *ERBB-2* gene amplification relates to the expression of p185^{HER-2} on a single cell basis. We have found that although p185^{HER-2} expression correlates with the *ERBB-2* copy number/cell, p185^{HER-2} expression correlates poorly with the *ERBB-2* copies:chromosome 17 copies ratio. Surprisingly, there was correlation between p185^{HER-2} expression and chromosome 17 copy number, suggesting that hyperploidy may be related to the p185^{HER-2} expression.

MATERIALS AND METHODS

Cell Lines. Human breast cancer cell lines, BT-474, SK-BR-3, MDA-453, and MCF-7 were obtained from the American Type Culture Collection (Rockville, MD) and grown according to their specifications. The four cell lines were characterized previously for *ERBB-2* gene amplification (10). For flow cytometric immunofluorescence measurements, cells were harvested either by trypsin or 25 mM EDTA in PBS (pH 7.2; Ref. 17). For slide-based immunofluorescence measurements, cells were cultured in slide chambers (Nunc, Inc., Naperville, IL). For BrdUrd (Sigma Chemical Co., St. Louis, MO), labeling cells were pulsed with 100 μM BrdUrd for 60 min. Cells were washed three times with PBS containing 1 mM $CaCl_2$ before immunofluorescence labeling.

Tumor. A biopsy from a node positive, T_2 tumor, was frozen immediately after resection. Imprint preparations were made after thawing by gently touch-

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[4] The abbreviations used are: FISH, fluorescence *in situ* hybridization; BrdUrd, bromodeoxyuridine; chr, chromosome; FI, fluorescence index.

ing the slide surface with tumor material. Slides were then fixed in 1% formaldehyde for 60 min at room temperature and subsequently fixed and stored in 70% ethanol. The autofluorescence of air-dried touch imprint preparations was too high for reliable immunofluorescence analysis. Fresh fixation of slides in 1% formaldehyde and subsequently in 70% ethanol reduced autofluorescence significantly.

Immunolabeling. For flow cytometry, unfixed trypsinized cells were incubated with 5 μg/ml mAb1 (Triton, Alameda, CA) raised against the extracellular domain of $p185^{HER-2}$, in the presence of 1% BSA on ice for 45 min, washed three times with PBS, and incubated with fluoresceinated rabbit antimouse IgG (1:100 dilution; Sigma) at 0°C for 45 min. After washing with PBS, cells were fixed in 1% formaldehyde solution and stored for not more than 3 weeks at 4°C before analysis.

For image analysis, cells were first fixed in 0.5% formaldehyde solution for 20 min at room temperature and in 70% ethanol at 4°C overnight. Cells on slides could be stored in ethanol at 4°C for not more than 2 months. Slides were then preblocked in 5% Carnation dry milk, 0.1% Triton X-100 in 4X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 10 min at room temperature. Staining was at room temperature for 45 min. Samples were first incubated with CB11 antibody (BioGenex, San Ramon, CA) specific to the intracellular domain of the $p185^{HER-2}$ protein, diluted (1:200) in the blocking buffer, washed twice with the blocking buffer, and incubated with fluoresceinated rabbit antimouse IgG (1:100; Sigma). After washing, samples were refixed in 1% formaldehyde solution in PBS and kept at 4°C for not more than 3 weeks before microscopic analysis. During this time, no significant deterioration of the fluorescence signal was observed.

To control for nonspecific staining, cells were preincubated with irrelevant monoclonal antibody of the same isotype before staining with fluorescein-conjugated rabbit antimouse IgG. We also compared immunofluorescence labeling of MDA-453 and SK-BR-3 cells harvested with either trypsin or 25 mM EDTA in PBS. Trypsinization caused a 10–15% loss in fluorescence intensity as compared to cells harvested with 25 mM EDTA (data not shown). Because this loss was not significant, and the two other cell lines could not be harvested with 25 mM EDTA, we used trypsin to harvest cells for flow cytometric analysis. Results from monoclonal antibody (mAb1) raised against the extracellular domain of $p185^{HER-2}$ protein were similar to those from monoclonal antibody (CB11) raised against the intracellular domain of the protein. With mAb1, prefixation was unnecessary, resulting in lower nonspecific binding.

Flow Cytometry. Cell suspensions were filtered through a 35-μm nylon mesh to remove aggregates before flow cytometric analysis. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW argon laser (488 nm) and pulse-width doublet discrimination. A total of 10,000 events were recorded in list mode after logarithmic amplification of the fluorescence signal.

Digital Image Analysis. The fluorescence of cells stained on slides was analyzed by using a digital image analysis system based on a Zeiss Axioplan microscope equipped with the MicroImager 1400 Digital camera (Xillix Technologies Corp., Vancouver, British Columbia, Canada). Images were captured through a fluorescein excitation filter, beam splitter, and emission filter by using a ×20, NA: 0.5 Plan Neofluar objective. Images were processed and quantitatively analyzed with a Sun IPX workstation using Scil-Image software (National Research Institute, Delft, The Netherlands). Local background fluorescence was determined for each image, and the average autofluorescence of the isotypic control cells was subtracted from the total fluorescence intensity of labeled cells. The FI was defined as a ratio of the corrected total fluorescence intensity of labeled cells to the mean autofluorescence of the isotypic control cells.

DNA Probes and Probe Labeling. Two contiguous *ERBB-2* cosmid clones (cRCNeu1 and cRCNeu4), together spanning 55 kb of genomic DNA (10), were used in combination with a probe specific for the chromosome 17 pericentromeric sequence (p17H8; Ref. 18) for two-color FISH analysis. Probes were directly labeled with fluorescein-11-dUTP or tetramethylrhodamine-11-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation by using commercially available kits (Bethesda Research Laboratories, Gaithersburg, MD).

Fluorescence *in Situ* Hybridization and Staining for BrdUrd. Dual analysis of gene copy number and protein expression was done as a two-stage procedure. Slides were first stained for protein expression and fluorescence images acquired; they were then hybridized for gene copy number and scored after relocating cells analyzed previously. After immunofluorescence analysis, slides were refixed in methanol:acetic acid (3:1; Carnoys solution) and air dried. FISH was performed as described previously (19) with modifications. Briefly, cells on slides were denatured in 70% formamide-2X SSC at 73°C for 3.0 min, dehydrated in graded ethanols, treated with 0.25 μg/ml proteinase K (Sigma) in 20 mM TRIS buffer (pH 7.5) containing 2 mM $CaCl_2$ for 7.5 min at 37°C, and again dehydrated. The hybridization mixture was denatured at 73°C for 5 min, reannealed for 30 min at 37°C, and applied to warmed slides. Ten μl of hybridization mixture contained 6 ng of fluoresceinated chromosome 17 centromeric probe, 34 ng of rhodaminated *ERBB-2* probe, and 10 ng of unlabeled, sonicated human placental DNA (Sigma) in 50% formamide, 2X SSC, and 10% dextran sulfate. Hybridization was overnight at 37°C. Slides were washed three times for 10 min each in 55% formamide-2X SSC, once in 2X SSC at 45°C, and once in 2X SSC at room temperature. Nuclei were counterstained by using 4′,6-diamidino-2-phenylindole hydrochloride (Molecular Probes, Eugene, OR) at 0.01 μg/ml in antifade solution (20).

Simultaneous detection of BrdUrd incorporation and dual FISH staining was performed with three fluorescent dyes (fluorescein- and rhodamine-labeled probes and a Cascade Blue-conjugated antibody; Ref. 21). Cells and probes were denatured and hybridized as described above. After washing, slides were preblocked in 5% Carnation dry milk and 0.1% Triton X-100 in 4X SSC for 10 min at room temperature. All staining reactions were at room temperature for 30 min. Slides were incubated with IU4 mouse anti-BrdUrd (1:400; Caltag, La Jolla, CA), diluted in blocking buffer, washed twice with blocking buffer, and incubated with Cascade Blue-antimouse IgG (1:300; Molecular Probes), and coverslipped with antifade solution alone.

Scoring of Interphase Nuclei. Cells analyzed previously for cell surface expression of $p185^{HER-2}$ protein were relocated on the basis of their coordinates and scored for chromosome 17 and *ERBB-2* signals by using a ×100 NA:1.3 Plan Neofluar oil immersion objective and a computer-controlled stage. *ERBB-2* doublets were counted as separate signals. Broken, torn, squashed, smeared, or overlapping nuclei were ignored. Each hybridization was accompanied by a control hybridization using normal lymphocytes. The scoring results were expressed both as an absolute *ERBB-2* copy number/cell and as the *ERBB-2* copy number relative to the 17 centromere copy number.

Three color images were acquired by using the digital imaging analysis system described previously. A triple-band-pass beam splitter and emission filters were used (22). Excitation of each fluorochrome was accomplished by using single-band-pass excitation filters in a computer-controlled filter wheel. This made it possible to collect sequential, properly registered images of the three fluorochromes (4′,6-diamidino-2-phenylindole hydrochloride or Cascade Blue, fluorescein, and rhodamine). The three-color images were processed with a Sun IPX workstation using Scil-Image software for pseudocolor display.

Statistical Analysis. Significance levels for differences in gene copy number between the $p185^{HER-2}$ bright and total cell populations were determined by contingency table analysis.

RESULTS

***ERBB-2* Amplification and Expression in Breast Cancer Cell Lines.** Four breast cancer cell lines, MCF-7, MDA-453, SK-BR-3, and BT-474, known to have various levels of amplification of the *ERBB-2* gene (10) were studied for distribution of *ERBB-2* gene copy number and chromosome 17 centromere copy number (Fig. 1). Amplification of the *ERBB-2* gene can be expressed as copy number/cell or as copy number relative to chromosome 17 copy number. Using a relative measure is especially important for those cell lines that are aneusomic for chromosome 17. Amplification of *ERBB-2* gene was observed in MDA-453, SK-BR-3, and BT-474 cell lines, using either the definition of amplification as total *ERBB-2* copies/cell or the ratio of *ERBB-2* copy number to chromosome 17 copy number. There was marked heterogeneity for *ERBB-2* copy number, chromosome 17 copy number, and their ratios in the three cell lines with *ERBB-2* amplification. In MCF-7, the *ERBB-2* gene was deleted (*ERBB-2* gene copy number was less than the chromosome 17 copy number/cell) and there was less heterogeneity in *ERBB-2* gene copy number/cell and in the

ERBB-2:chromosome 17 ratio. The mean values and the SDs of the copy number distributions are summarized in Table 1.

We next characterized the expression levels of the *ERBB-2* gene product p185^{HER-2} by flow cytometry. Fig. 2 shows the fluorescence intensity histograms of the four cell lines labeled with mAb1 against p185^{HER-2}. Heterogeneity of expression of p185^{HER-2} was similar in the four cell lines. The MCF-7 cell line was the least positive, only twice background, whereas the BT-474 cells were the most positive.

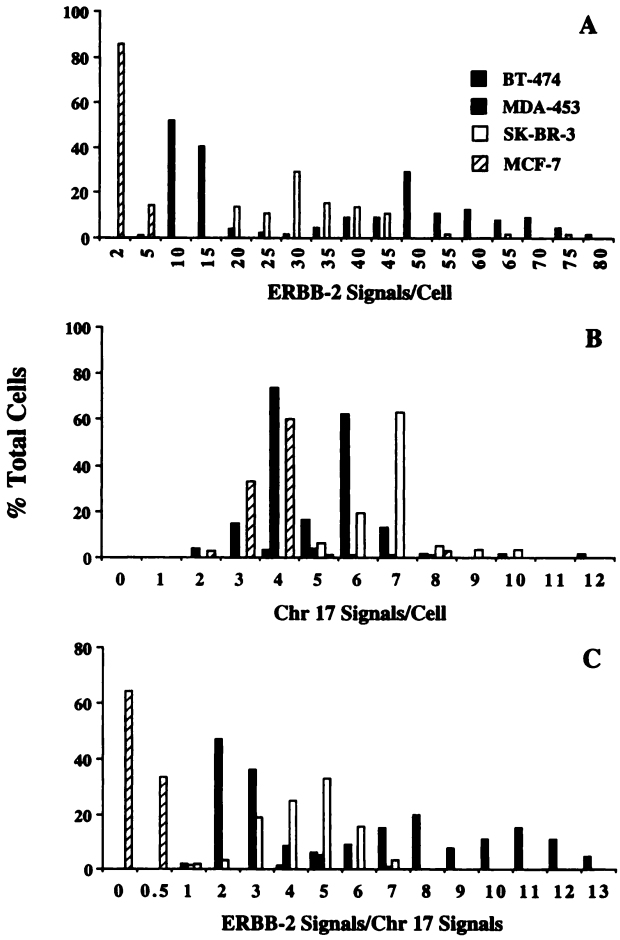

Fig. 1. Number of *ERBB-2* and chromosome 17 centromere copies in four breast cancer cell lines. *A*, frequency distribution of *ERBB-2* signals/cell; *B*, chromosome 17 signals/cell; *C*, *ERBB-2*:chromosome (*Chr*) 17 ratio. The values along the *abscissa* represent the lower limits of the range of values for each category. At least 100 cells were scored to create the distribution histograms. The mean values and their SDs are summarized in Table 1. Note the wide heterogeneity present in all but the MCF-7 distributions.

Table 1 *ERBB-2 amplification and expression in breast cancer cell lines*

Breast cancer cell lines	ERBB-2[a]	Chr 17[b]	ERBB-2/Chr 17	FI[c]
MCF-7	2.2 ± 0.5[d]	3.8 ± 1.0	0.6 ± 0.2	20 ± 9
MDA-453	11.0 ± 3.9	4.1 ± 1.6	2.8 ± 1.0	186 ± 75
SK-BR-3	31.0 ± 9.0	6.9 ± 1.0	4.5 ± 1.2	326 ± 114
BT-474	52.0 ± 11.3	6.0 ± 1.1	9.0 ± 2.3	549 ± 165

[a] *ERBB-2* copy number/cell.
[b] Chr, chromosome 17 copy number/cell.
[c] Mean fluorescence intensity determined from flow cytometric histograms.
[d] Data expressed as mean, ±SD.

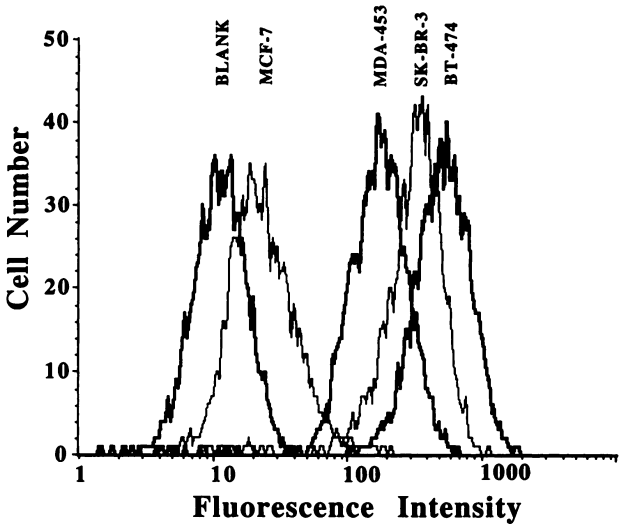

Fig. 2. Frequency distribution of fluorescence intensity after immunofluorescence staining for p185^{HER-2}. Trypsinized cells were labeled with mAb1 raised against the extracellular domain of p185^{HER-2} and then with fluorescein-conjugated rabbit antimouse IgG. An irrelevant primary antibody of the same isotype, followed by fluorescein-conjugated rabbit antimouse IgG, was used for the blank control (SK-BR-3 cells). The mean values of these distribution curves are summarized in Table 1. Note that the level of heterogeneity (width of the intensity profiles on this log intensity scale) is similar in the four cell lines, although the absolute amount of p185^{HER-2} varies greatly from line to line.

The mean values and the SDs of the fluorescence intensity histograms are summarized in Table 1. The mean fluorescence intensity was strongly correlated with the mean *ERBB-2* copy number/cell (r = 0.99; Table 1). A strong correlation was also observed between the mean protein expression and mean *ERBB-2*:chromosome 17 ratio (r = 0.99), whereas there was a weaker correlation with average chromosome 17 copy number (r = 0.75).

***ERBB-2* Gene Expression and Amplification on a Single Cell Basis.** Protein expression and copy number were measured in the same individual cells to study their correlation on a single cell basis. This was especially relevant given the wide range in both copy number and immunofluorescence observed (Figs. 1 and 2). Immunofluorescence intensity of individual SK-BR-3 and MDA-453 cells was studied by image microscopy, and the same cells were identified and scored for *ERBB-2* gene and chromosome 17 copy number after dual FISH labeling. The fluorescence intensity was too low in MCF-7 to perform quantitative image cytometry, and BT-474 cells could not be separated from each other during image analysis because of their piled-up growth pattern.

Correlated measurement of p185^{HER-2} expression and *ERBB-2* copy number was performed in the same cells by consecutive analysis (Fig. 3). The fluorescence images of cells displayed in Fig. 3*B* are shown after double-target hybridization in Fig. 3*C*. The green signals correspond to chromosome 17 centromere, and the red signals to *ERBB-2* signals. The heterogeneity of p185^{HER-2} expression in SK-BR-3 cells by image microscopy (Fig. 3*A*) was similar to that found by flow cytometry (Fig. 2).

The linked analysis of p185^{HER-2} expression and *ERBB-2* gene amplification in SK-BR-3 cells is shown in Figs. 4 and 5. Note the use of a FI for these measurements, rather than absolute intensity (as was used for the flow measurements), in order to control for the increased levels of autofluorescence in these fixed samples. *ERBB-2* copy number showed a significant correlation with protein expression on a cell-by-cell basis. The correlation was stronger using absolute *ERBB-2* copy number/cell (Fig. 4*A*) than when using a relative

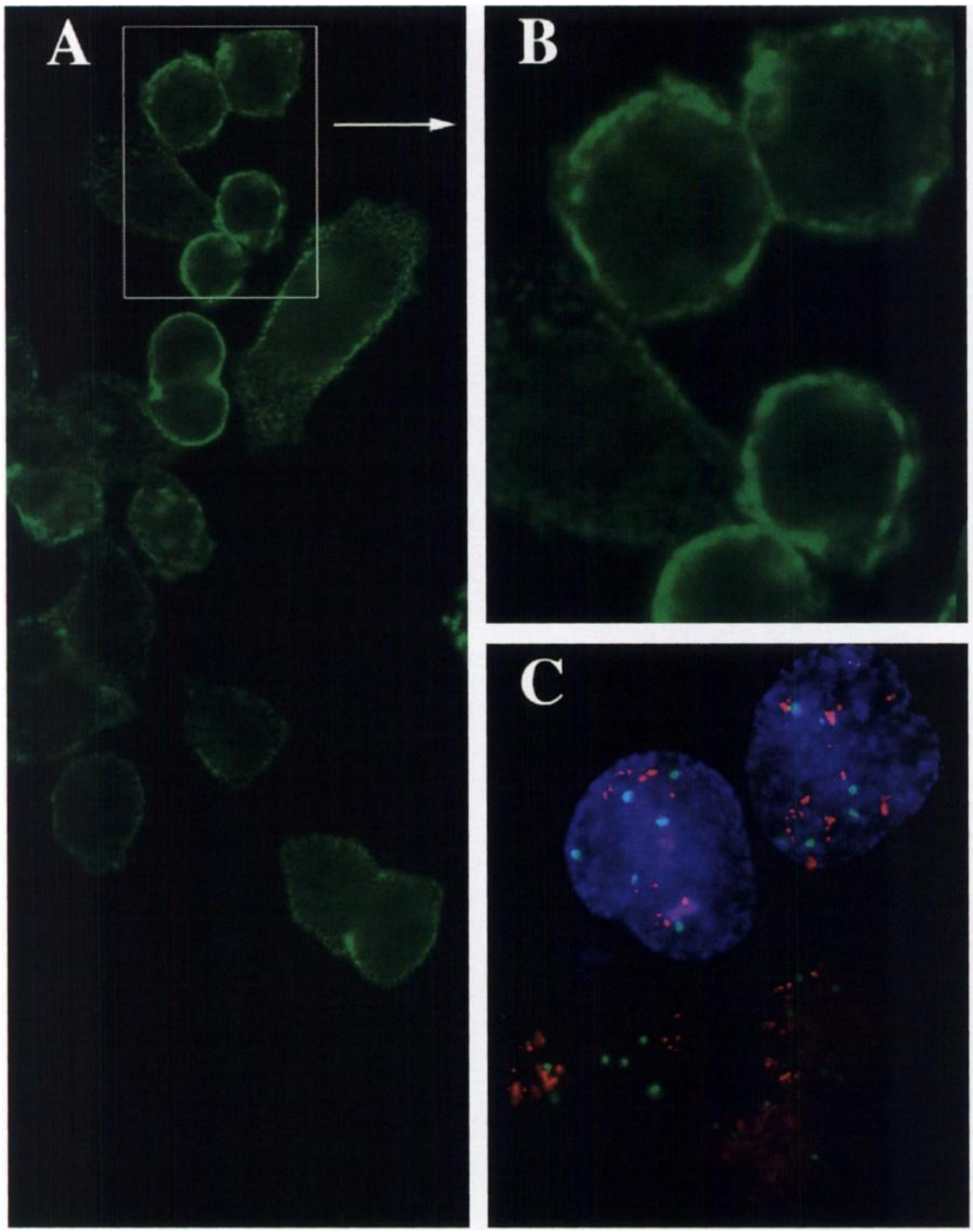

Fig. 3. Linked detection of $p185^{HER-2}$ expression and gene amplification in individual cells. *A*, SK-BR-3 cells display immunofluorescence staining for $p185^{HER-2}$ expression (×20 objective) after staining with mAb1. *B*, computer magnification (×5) of the rectangle in *A*. *C*, FISH detection of *ERBB-2* (*red*) and chromosome 17 centromeres (*green*) in identical cells shown in *B* (×100 objective). Cells were refixed after immunofluorescence labeling and denatured and hybridized with directly labeled *ERBB-2* and chromosome 17 centromere-specific probes. Not all signals are visible in this image because the plane of focus is thinner than the specimen. Anti-BrdUrd labeling (*blue*) is positive in the top cells. These are pseudocolor, contrast-enhanced digital images.

measure of *ERBB-2* amplification (*ERBB-2*:chromosome 17 copy number ratio; Fig. 4*B*). There was also a correlation seen between $p185^{HER-2}$ expression and copy number of chromosome 17 (Fig. 4*C*), perhaps due to an second association between aneuploidy and *ERBB-2* amplification.

A subpopulation of cells was seen, which stained especially brightly for $p185^{HER-2}$. To test whether this was due to genetic heterogeneity or to phenotypic dispersion, the genetic composition of "bright" cells (with more than four times more fluorescence intensity than the nonspecific staining of isotypic control cells) was analyzed as a separate group. We compared the distribution of *ERBB-2* copy number (Fig. 5*A*), *ERBB-2*: chromosome 17 copy number ratio (Fig. 5*B*) and the chromosome 17 copy number (Fig. 5*C*) of bright cells to an unselected population and found that these differences were all highly significant.

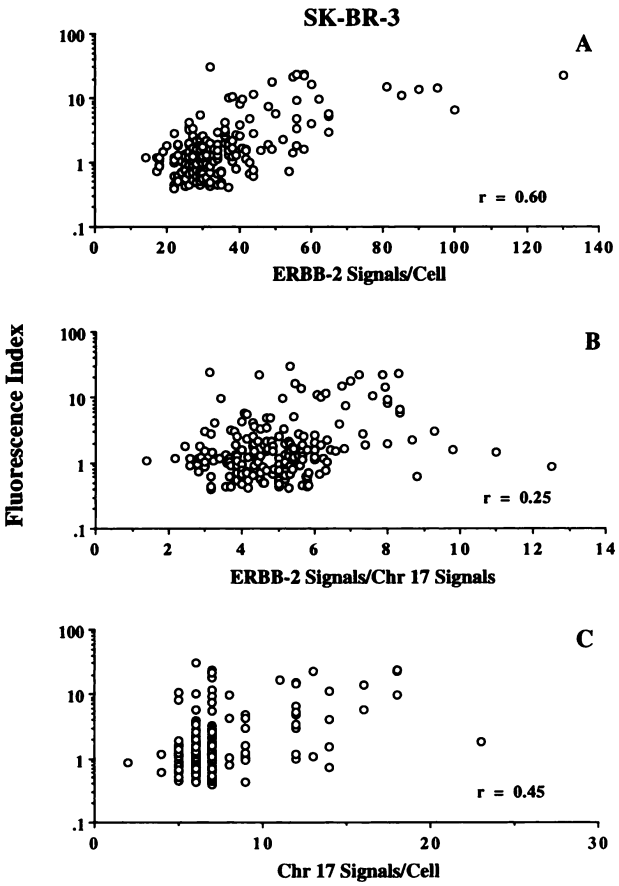

Fig. 4. *ERBB-2* gene expression and amplification in single SK-BR-3 cells. Expression level of $p185^{HER-2}$ protein (FI) plotted against: *A*, *ERBB-2* copy number; *B*, *ERBB-2*:chromosome 17 ratio; and *C*, chromosome 17 copy number. Cells were labeled with antibody (CB11) against the intracellular domain of $p185^{HER-2}$ protein. Data from 184 cells are shown. There is a good correlation between $p185^{HER-2}$ expression and copy number of either *ERBB-2* or chromosome 17 centromere (*A* and *C*). The correlation between $p185^{HER-2}$ expression and *ERBB-2*:chromosome 17 copy number ratio was weaker (*B*).

The results in MDA-453 cells were similar to SK-BR-3 cells for *ERBB-2* gene amplification and protein expression (Figs. 6 and 7). However, there was no correlation between protein expression and *ERBB-2*:chromosome 17 copy number ratio (Fig. 6*B*), and the distribution of *ERBB-2*:chromosome 17 ratio of bright cells did not differ significantly from the unselected population (Fig. 7*B*). In both cell lines, the centromere 17 copy number was high (two or three times the average copy number) in >50% of the bright cells (expressing a high level of $p185^{HER-2}$), whereas <2% of the unselected population had a high chromosome 17 copy number.

Relationship between DNA Synthesis and *ERBB-2* Gene Expression and Amplification. We next addressed the issue of whether the bright cells having high $p185^{HER-2}$ expression and high chromosome 17 copy number were proliferatively active. SKBR-3 cells were pulse labeled with BrdUrd, $p185^{HER-2}$ expression was determined before fixation, and then BrdUrd incorporation and dual-color FISH were detected simultaneously (for demonstration see Fig. 3*C*) Correlation between *ERBB-2* gene amplification and protein expression in these cells (data not shown) was similar to that found in prefixed cells (Figs. 4 and 5). When BrdUrd-positive cells (cells in S phase) were compared with BrdUrd-negative cells, there was no significant difference ($P = 0.29$) in the average fluorescence intensity. S-phase cells had a higher average *ERBB-2* gene copy number and *ERBB-2*:chromosome 17 copy number ratio than did non-S-phase cells (43.7 *versus* 38.7 and 6.3 *versus* 5.0, respectively), perhaps because doublets forming during DNA synthesis were scored as two separate gene copy numbers as described in "Materials and Methods." The labeling index of the whole cell population (39.7% of 224 cells) and for cells with >10 chromosome 17 copies (38.3% of 60 cells) did not differ.

***ERBB-2* Gene Expression and Amplification on a Primary Tumor Sample.** The results of consecutive analysis of $p185^{HER-2}$ expression and *ERBB-2* gene amplification in primary tumor cells (case no. B372) are shown in Figs. 8 and 9. Positive correlations were found between $p185^{HER-2}$ expression and *ERBB-2* gene copy number (Fig. 8*A*), the *ERBB-2*:chromosome 17 ratio (Fig. 8*B*), and the chromosome 17 copy number (Fig. 8*C*). There were significant differences in the distribution of the *ERBB-2* copy number (Fig. 9*A*), the ratio of *ERBB-2*:chromosome copy number (Fig. 9*B*), and the chromosome 17 copy number (Fig. 9*C*) when bright cells were compared to the unselected population. In general, the correlation patterns observed in this touch imprint preparation were similar to those observed in tumor cell lines.

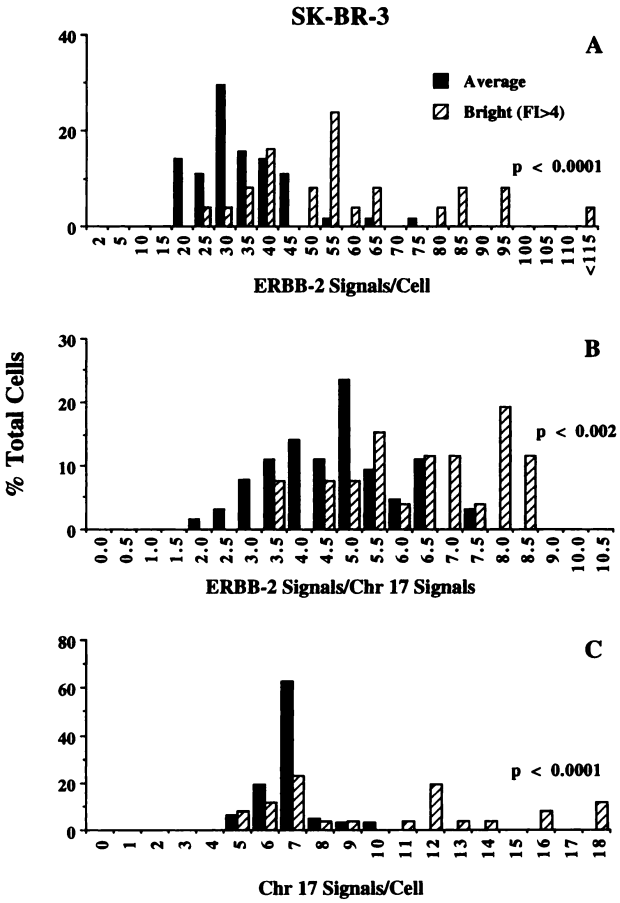

Fig. 5. *ERBB-2* copy number (*A*), *ERBB-2*:chromosome (*Chr*) 17 ratio (*B*), and chromosome 17 copy number (*C*) in unselected and in highly $p185^{HER-2}$-expressing (FI > 4) SK-BR-3 cells. Distributions of bright cells were determined from cells plotted in Fig. 4. Cells that expressed $p185^{HER-2}$ at a high level had significantly more copies of *ERBB-2* and chromosome 17, and a higher ratio of the two, than an unselected population.

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