Genetic Alterations in ERBB2-amplified Breast Carcinomas\textsuperscript{1,2}

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ABSTRACT

Amplification of the ERBB2 oncogene has recently received attention as a target for antibody-based therapies and as a predictor of response to adjuvant chemotherapy. Modification of treatment strategies based on ERBB2 status has led to further interest in the genetic alterations that accompany ERBB2 gene amplification or overexpression. In this study, chromosome alterations that are associated with ERBB2 amplification were defined by comparative genomic hybridization (CGH). Additionally, fluorescence in situ hybridization (FISH) was used to validate gene amplification, and protein expression was detected immunohistochemically. ERBB2-amplified tumors as detected by FISH, immunohistochemistry (IHC), or CGH had twice as many CGH-defined chromosomal alterations (means of 11.8, 11.0, and 12.7, respectively) as the nonamplified tumors (means of 6.8, 7.0, and 5.6, respectively). ERBB2 positivity correlated with the total number of genetic events. A wide spectrum of copy number gains and losses was seen by CGH in all of the tumors. An increased number of losses of 18q and gains of 20q was found in ERBB2-positive tumors. Other common aberrations for all of the tumors were copy number gains of 1q (58%), 8q (52%), 20q (30%), and losses of 18q (39%), 13q (39%), and 3p (33%). A high degree of concordance was observed among the three methods in 33 primary breast cancers. The concurrence for ERBB2 detection between FISH and IHC was 90%, between FISH and CGH was 82%, and between IHC and CGH was 84%. This study shows that breast tumors showing erbB2 overexpression or gene amplification are genetically distinct from erbB2-negative tumors. These differences may relate to the mechanisms underlying altered response to adjuvant therapies and may define the responsiveness to erbB2-directed immunotherapy.

INTRODUCTION

Amplification of the ERBB2 oncogene is found in 20–30% of human breast cancers (1). Several lines of evidence suggest that the amplification of ERBB2 and consequent protein overexpression play an important role in cancer progression. Many studies have shown an association between ERBB2 amplification and aneuploidy, rapid cell proliferation, low estrogen-receptor content and high histological grade (2–5). ERBB2 amplification has also been implicated as a predictor of altered response to doxorubicin therapy (5–7) and, in many studies, an indicator of poor prognosis in node-positive patients (2). Recent IHC\textsuperscript{4} studies by Thor et al. (7) and Paik et al. (5) showed that patients with erbB2-overexpressing tumors had a better clinical outcome after doxorubicin-containing therapy than those patients with nonoverexpressing tumors. Modification of treatment strategies based on ERBB2 status has led to further interest in defining the genetic alterations that accompany ERBB2 gene amplification or overexpression (8–11).

It is not clear to what extent breast tumors with ERBB2 amplification and/or overexpression contain other genetic aberrations. The coamplification of MYC and ERBB2, as detected by FISH, has been associated with larger tumor size and aneuploidy (10). With the introduction of CGH, genome-wide screening for gene copy number aberrations has become possible (12–15). Amplification of another region on chromosome 17, at 17q22–q24, was detected in primary breast cancers and cell lines by CGH (16). The aim of the present study was to define ERBB2-associated chromosomal alterations and to define the sensitivity of CGH compared with FISH and IHC in detecting ERBB2 status.

MATERIALS AND METHODS

Samples. Four established breast cancer cell lines (BT-474, SK-BR-3, MDA453, and MCF-7) were obtained from the American Type Culture Collection (Manassas, VA) and cultured under standard conditions. Thirty-three breast carcinomas were acquired from the University of California-San Francisco Medical Center (San Francisco, CA). All of the studies received prior approval from the University of California-San Francisco Institutional Review Board. None of the patients had received preoperative chemo- or radiotherapy, and all of the tumor samples were obtained from the primary tumor site. Formalin-fixed sections were reviewed for histopathological criteria according to the Scarff-Bloom Richardson grading system. All of the tumors were invasive ductal carcinomas. Twelve cases were...
selected based on the presence of ERBB2 amplification as detected by FISH, and 21 cases had normal ERBB2 copy number.

**FISH.** Tumor touch preparations were made by lightly pressing fresh tumor samples onto cleaned microscope slides. A parallel slide was stained by Giemsa and was reviewed to determine the fraction of malignant cells present. Two-color FISH was performed as described previously (9) using two contiguous ERBB2 cosmid clones (cRNeu1 and cRNeu4) and a probe specific for a pericentromeric sequence on chromosome 17 (p17H8). Each tumor cell was scored for the number of centromeric and ERBB2 signals. At least 100 tumor cells were examined for each sample. Amplification of ERBB2 was determined by the ratio of the average number of ERBB2 signals relative to the average number of chromosome 17 centromere signals in each sample. Amplification was defined as a ratio of 1.5 or more (representing at least one extra copy of ERBB2 in diploid cells). Probe hybridization efficiency was tested in every experiment using normal peripheral blood lymphocytes. In all of the experiments, more than 90% of lymphocyte nuclei showed two signals for both ERBB2 and chromosome 17 centromere probes.

**CGH.** CGH was performed using DNA isolated from frozen primary tumors as described previously (13, 17). Briefly, genomic DNA was extracted from freshly frozen tumor samples and normal peripheral blood lymphocytes. DNA was nick-translated using fluorescein-12-dUTP (DuPont, Boston, MA) for tumor DNA and Texas Red-5-dUTP (DuPont) for normal female reference DNA. In each reaction, 250 ng of labeled reference and tumor DNA and 10 µg of unlabeled Cot-1 DNA (Life Technologies, Gaithersburg, MD) were hybridized to normal lymphocyte metaphase preparations for 48 h. After hybridization, slides were washed and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Quantitative analysis of relative DNA sequence copy number was done as described previously (18). Green:red ratios above 1.2 were considered indicative of DNA copy number gain, and ratios less than 0.85 were indicative of copy number loss. In accordance with our previous studies (19), copy number gains spanning less than an entire chromosome arm (regional gains) were considered indicative of gene amplification. ERBB2 amplification by CGH was defined by a regional gain involving 17q12, and also by gains of the entire 17q arm.

**IHC.** Formalin-fixed tissue sections were stained for erbB2 overexpression with the monoclonal antibody CB11 (BioGenex, San Ramon, CA). Antigen retrieval by microwaving was not necessary for this antibody. After deparaffinization, slides were incubated overnight at 4°C with a 1:50 dilution of the antibody. Antibody staining was observed using biotin-antimouse (Vector Laboratories, Burlingame, CA) and streptavidin (BioGenex), followed by diaminobenzidine. Sections were then counterstained with hematoxylin. Cell lines BT474 and MCF7 were used as positive and negative controls, respectively. Cases were scored as positive if any tumor cells showed definitive membrane staining. Cytoplasmic staining alone was scored as negative.

**Statistics.** The Statview computer analysis program was used to perform ANOVA and χ² tests to compare the three methods and to correlate ERBB2 status with tumor aggressiveness.

**RESULTS**

**ERBB2 Amplification by CGH.** CGH detection of ERBB2 amplification was tested in breast cancer cell lines with known ERBB2 amplification status (Fig. 1). Positive control cell lines, BT-474 and SK-BR-3, showed clear copy number gains at 17q12, the chromosomal locus of ERBB2. These cell lines had ERBB2:centromeric copy number ratios of 9.0 and 4.5, respectively as detected by FISH. Neither the MDA453 cell line, with an ERBB2 copy number ratio of 2.8 and overexpression by IHC, nor the MCF-7 cell line, with an ERBB2 copy number ratio of 0.6 and no expression by IHC, showed any detectable gain by CGH at 17q12. Interestingly, all of the four cell lines showed gains distal to 17q12.

Fourteen cases showed copy number gain at 17q12, including 2 cases with whole arm gains. Ten of the 12 tumors with FISH ERBB2 amplification showed a 17q12 copy number gain. As in the cell lines, clinical tumor samples showed highly variable profiles at 17q (Fig. 1). The highest peak of amplification at proximal 17q was at the q12 region in eight tumors. An amplification peak was detected slightly outside 17q12 in two tumors (B336, B413). The size of the chromosomal region showing copy number increase was highly variable. In one case the amplicon extended to 17p (B412). A regional copy number gain in the distal chromosome arm involving 17q22–q24 was seen in five tumors (B289, B297, B372, B409, and B412). Three additional tumors (B424, B413, and B310) also had an increased ratio in this distal region, but it was unclear whether this reflected a copy number gain of the entire 17q arm as opposed to a regional gain.

**ERBB2 Amplification by FISH.** ERBB2 copy number was detected by FISH in touch preparations from thirty-three breast tumors. An ERBB2:chromosome 17 centromere ratio greater than 1.5 was used to define amplification. Twelve cases were ERBB2-amplified by this definition. The mean copy number in the amplified tumors was 14.0, ranging from 1.5 to 40.9.

**ErbB2 Overexpression by IHC.** Definitive membranous staining in any tumor cell was considered positive. Twelve cases showed erbB2 overexpression by this definition. The mean fraction of cells staining positive was 66%. Only 1 of the 12 positive cases showed expression in less than 10% of the cells. Twenty cases showed no cells staining positively.

**Genetic Aberrations Associated with ERBB2 Amplification.** The total number of changes detected by CGH in the ERBB2-amplified tumors was higher than in the nonamplified groups using all three of the methods (Table 1). When gains and losses were analyzed separately, the numbers were too small to detect a statistically significant difference.

The most common aberrations for all of the tumors were copy number gains of 1q (58%), 8q (52%), and 20q (30%), and losses of 18q (39%), 13q (39%), and 3p (33%). The gain of chromosome 20q, and the loss of 18q, were significantly greater for the ERBB2-amplified sets than in the nonamplified group (P < 0.05 using CGH). Gains of 16p and 17q22–q24 and losses on 8p tended to be more common in the ERBB2-amplified tumors than in the control group. Most other genetic aberrations,
**Fig. 1** ERBB2 by CGH, FISH, and IHC. DNA ratio profiles of chromosome 17 for four breast cancer cell lines and 9 primary breast cancers. CGH profiles are expressed as a mean of four chromosomes (thick line) ± one SD (thin lines). Detection limits for ratio changes (0.85–1.2) are shown with dotted lines. ERBB2:FISH ratio was calculated as the mean of the ratios of the total number of ERBB2 signals divided by the number of centromere 17 signals per cell. Case B412 had no available (N/A) IHC results.
including 1q+, 13q−, and 3p−, were approximately equal in the \( ERBB2 \)-amplified and -nonamplified groups. All of the chromosome arms were involved at least once in all of the tumors with CGH aberrations (30 cases). The three tumors with no \( ERBB2 \) amplification by all three methods also showed no copy number aberrations by CGH.

Regional gains at loci distinct from 17q12 were found in 15 of 17 \( ERBB2 \)-amplified cases (by any of the three methods), and in 8 of 16 nonamplified cases. The most common regional gains in these 15 \( ERBB2 \)-amplified cases were on chromosome 1q (four cases), 8q (five cases), and 17q22–qter (six cases). The most common regional gains in the eight \( ERBB2 \)-nonamplified cases were on chromosome 8q (three cases) and 17q22–qter (three cases).

Comparison of FISH, IHC, and CGH. The concordance among the three detection methods is shown in Table 2. Concordance was 91\% between IHC and FISH (29 of 32 cases), 82\% for FISH and CGH (27 of 33 cases), and 84\% for IHC and CGH (27 of 32). A good correlation \((r = 0.95)\) of IHC and FISH for each case was observed. Nine cases were negative for \( ERBB2 \) by all of the three methods, and 16 were positive for \( ERBB2 \) by all of the three methods.

Clinical Associations. \( ERBB2 \)-positive tumors were represented by a tumor phenotype different from the \( ERBB2 \)-negative tumors (Table 3). The \( ERBB2 \)-positive tumors tended to be larger, ER-negative, node-positive, and more likely to recur.

DISCUSSION

The introduction of CGH has markedly expanded our knowledge of genetic aberrations in solid tumors. The total number of aberrations by CGH is an indicator of the genetic “grade” of a tumor (19). In the current study, breast tumors with \( ERBB2 \) amplification showed a significantly higher number of aberrations than nonamplified tumors, indicating that erbB2-altered cancers are, on average, genetically more advanced.

As in previous allelotyping (20, 21) and CGH studies (13, 14, 19), we found a wide variety of chromosomal aberrations in primary breast cancers. Although most of the frequently observed aberrations were equally common in \( ERBB2 \)-amplified and -nonamplified tumors, gains of 20q and losses of 18q were more common in \( ERBB2 \)-amplified tumors. The gain of 20q is particularly interesting, because we have previously demonstrated the prognostic significance of 20q gain in breast cancers (19, 22, 23). These findings suggest that prognostic associations of \( ERBB2 \) should be stratified for chromosome 20q status in multivariate statistical analyses. The loss of 18q, the locus of several tumor suppressor genes (24), suggests the need for characterization of this region in \( ERBB2 \)-amplified tumors.
described previously in cell lines (16, 17), these tumors identify an amplicon at 17q22– q24, clearly distinct from ERBB2 amplification at 17q12. This distal 17q amplification has not been characterized fully in clinical tumors, although candidate oncogenes have been described (25).

We examined the sensitivity of CGH to detect amplification of ERBB2. Our early experience suggested that small amplicons may not be accurately detected by CGH in clinical tumor samples. Current results show that ERBB2 oncogene amplification was accurately detected by CGH, and that CGH correlated with both IHC and FISH for ERBB2 detection. In this study, we included whole chromosome 17q arm gains as a measure of ERBB2 amplification. This prevented amplicons going undetected by CGH when they are present within whole arm changes, as has been reported for chromosomal arms 17q and 20q (16, 22).

The concordance between FISH and CGH in detecting ERBB2 amplification was similar to that reported among other techniques such as Southern hybridization, FISH, and IHC (9, 11, 26). FISH has been used previously to confirm CGH profiles for unknown oncogenes in breast cancer on chromosomes 17q and 20q (14, 16, 23). CGH has the disadvantage that the number of gene copies (level of amplification) cannot be determined directly. In our experience, the maximum tumor:reference DNA ratio never exceeds four, although the actual gene copy number (determined by FISH) may be up to 40-fold greater than other loci. This may be explained in part by nonlinear quantitation. In addition, it might have been influenced by tumor heterogeneity and normal cell contamination. Total tumor DNA was used for CGH analysis, and, although efforts were taken to minimize normal cell contamination, tumor heterogeneity could not be controlled for. FISH gene copy number measurement allows the exclusion of normal cells and limits the scoring to signals within individual tumor cells. This could explain the results for case B424, in which FISH detected a 40-fold amplification of ERBB2, but the CGH ratio was only 2.0.

In this study, we have confirmed previous reports that erbB2 overexpression, as measured by IHC, correlates with increased copy number by FISH (11, 27). We showed a correlation between erbB2 overexpression and ERBB2:centromere 17 ratio copy number \( r = 0.78 \). An even better correlation was observed when looking at overall ERBB2 copy number \( r = 0.95 \). This confirms work done in cell lines by Szollosi et al. (27), in which FISH copy number was measured in individual cells simultaneously with the degree of protein expression by immunofluorescence, showing a strong correlation between ERBB2 copy number and degree of protein expression. The association between intensity of erbB2 staining and ERBB2:centromere 17 copy number ratio was not as strong in that study.

The best approach for scoring of ERBB2 signals in tumor samples is still controversial (absolute count versus ratio-to-centromere count) and should be studied further in large tumor cohorts using standardized protocols.

We have shown that breast tumors showing erbB2 overexpression or gene amplification are genetically distinct from erbB2-negative tumors. These differences may relate to the mechanisms underlying altered response to adjuvant therapies, and may define the responsiveness to erbB2-directed immuno-
therapy. Future studies will apply new technologies such as tissue and DNA micro-arrays (28, 29) to these questions to allow screening of ERBB2 positive tumors for associated gene alterations.

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