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Available at: https://works.bepress.com/joseph_lucke/32/
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Received for publication December 16, 1994; accepted May 16, 1995

Studies of amplification and/or overexpression of c-myc, HER-2/neu, and H-ras in breast cancer have shown that each is associated with a poor prognosis. The purpose of this study was to explore the possibility that there is a preferred sequence of amplification of these oncogenes in breast cancer. The frequencies of amplification and patterns of co-amplification of c-myc, HER-2/neu, and H-ras were studied in a group of 84 breast cancers. The data suggested a preferred sequence of amplification that consisted of c-myc amplification-HER-2/neu amplification-H-ras amplification. This model was supported by loglinear analysis. In addition, the levels of amplification of JC-A, a DNA fragment newly isolated from a patient with advanced breast cancer, were studied in 61 of these cases. The data suggested that JC-A amplification occurred early. Loglinear analysis supported a model in which JC-A amplification occurred either before or after c-myc amplification but was unrelated to Her-2/neu or ras amplification.

Key terms: Oncogene amplification, oncogene overexpression, breast cancer, genetic evolution

Abnormally activated oncogenes have been implicated in the development and progression of human breast cancer. These include amplification and/or overexpression of c-myc (4-6,19,33), HER-2/neu (9,27), EGF receptor (21,31), cyclin D/PRAD1 (18,22) and cathepsin D (30). Loss or alteration in function of tumor suppressor genes is also common. Of these, p53 (16,26), Rb (7) and nm23 (3) have been studied extensively. In addition to genetic alterations involving known oncogenes, gene amplification has been observed by cytogenetic techniques and comparative genomic hybridization at loci that do not correspond to those of known oncogenes (15,35). It is not known if these genetic changes occur in a predetermined sequence. Preferred sequences of genetic alterations have been demonstrated in colon cancer (10,32) and bladder cancer (25). In this paper, we sought to determine if oncogene alterations accumulate in a preferred order in human breast cancer as well.

The amplification and co-amplification of c-myc, HER-2/neu and H-ras were determined in a group of 84 human breast tumors. A subgroup of 61 tumors was also tested for amplification of JC-A, a novel amplified DNA fragment isolated from a patient with advanced breast cancer that has also been found to be amplified in other breast cancers as well. The relative frequencies of oncogene amplification in our studies and in the published literature suggested specific sequences of oncogene amplification, which were then subjected to loglinear analysis.

MATERIALS AND METHODS

Cell Samples

Eighty-four fresh breast tumor samples were obtained for study from the Department of Pathology at Allegheny General Hospital, Pittsburgh, PA, and Jameson Memorial Hospital in New Castle, PA. Seventy samples were from patients with apparently localized disease. Of these, 26 had no axillary node involvement, and 44 had documented axillary node metastases; there was no sampling of axillary nodes in 21 cases. Fourteen samples were obtained from patients with metastatic disease.

Tumor samples were transported to the laboratory on ice, usually within one to two hours after surgery. Samples were minced finely with scissors in Hanks Balanced Salt Solution (HBSS), filtered through 64 μm nylon mesh (Small Parts, Miami, FL) and washed once with HBSS. Cytospin slides (Shandon, Pittsburgh, PA) were prepared from the cell suspension; these later underwent histologic review to confirm the presence of tumor cells in the sample. Part of the cell suspension was fixed for use in other studies and the remainder was combined with...
the residual tumor mince for oncogene amplification studies.

**Cell Lines**

SKBR-3, a human breast adenocarcinoma cell line with HER-2/neu amplification, and COLO 320 DM, a human colon adenocarcinoma with c-myc amplification, were obtained from ATCC (Rockville, MD) for use as positive controls for HER-2/neu, H-ras or c-myc amplification.

**Isolation of the Amplified J-C-A Fragment**

Competitive DNA reassociation techniques were used to select for amplified MboI fragments (24) in breast tumor DNA obtained from a patient with advanced disease. A plasmid library was created using the multifunctional pSPORT1 vector (Life Technologies, Gaithersburg, MD) and DNA enriched for amplified sequences. One clone designated J-C-A detected amplified restriction fragments on Southern blot analysis. A 0.4 kb MboI fragment devoid of repetitive elements was used to probe dot blots of control (human placenta) and breast tumor DNA. The insert was partially sequenced and had no matches in the GenBank nucleotide sequence data base. It was mapped to chromosome 19 using a somatic cell hybrid panel (Corell Institute, Camden, NJ).

**Gene Amplification Studies**

DNA extraction. Breast tumor samples were placed in buffer containing 100 µg/ml proteinase K (Sigma Chemical Company, St. Louis, MO) and 1% SDS. Protein was then removed by sequential phenol-chloroform, chloroform extraction. High molecular weight DNA was obtained by spooling upon precipitation in absolute ethanol. Concentrations were determined colorimetrically by the Burton assay (8).

**Dot blot analysis.** Identical amounts of denatured DNA (14) from each sample (0.5 µg to 2.5 µg) were applied in duplicate to Nytran filters (Schleicher & Schuell, Keene, NH) with the aid of a dot blot manifold applied in duplicate to Nytran filters (Schleicher & Schuell, Keene, NH). Placental DNA served as a control for diploid copy number. SKBR-3 DNA and COLO 320 DM DNA were positive controls for HER-2/neu and c-myc amplification, respectively. Blots were sequentially probed for oncogene amplification (HER-2/neu, c-myc, H-ras and J-C-A) after stripping of blots between hybridizations. Complete removal of signal was verified on the Betascope 603 blot analyzer (Intelligenetics/Betagen, Framingham, MA). Hybridizations were carried out for 24-48 hours at 45°C in hybridization buffer (50% formamide, 5X Denhardt's reagent, 0.5% SDS, 6X SSPE) containing 32P-dCTP labeled probe.

The following inserts were labeled by random primer extension (13): a 9 kb Eco RI/Hind III fragment of pHSR-1 (c-myc), a 1.45 kb Eco RI fragment of pHER-436-1 (HER-2/neu), a 6.6 kb Bam H1 fragment of pUC EJ 6.6 (H-ras) and a 0.4 kb MboI fragment of J-C-A. Bacteria containing the appropriate plasmids (c-myc, HER-2/neu, and H-ras) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and amplified according to standard procedures.

To correct for differences in loading efficiency and integrity of DNA, blots were reprobed for total DNA using the BLUR8 (Alu) probe (Oncor, Gaithersburg, MD).

All signals were quantitated using the Betascope 603 blot analyzer (Intelligenetics/Betagen, Framingham, MA). Signals were compared to those of placental DNA which served as a control for diploid copy number. Any signal at least two-fold greater than that of placenta was classified as an increase in gene copy number.

**Statistical Analysis**

Loglinear models were used to investigate the postulated causal sequence among the oncogenes studied (2). The strategy was to construct a statistical model that represented the associations among the four oncogenes constrained by the assumed temporal sequence of amplification.

The use of loglinear analysis requires some additional explanation. The patterns of amplification among the four oncogenes form a 4-dimensional table. The standard $\chi^2$ analyses of 2-dimensional tables cannot utilize all the information contained in a 4-dimensional table. For example, the standard analysis can only test the independence of amplification frequencies between pairs of oncogenes, but cannot test the complete independence of amplification frequencies among the 4 oncogenes.

Loglinear analysis is an extension of the more familiar analysis of two-dimensional contingency tables to 3-, 4-, and higher-dimensional tables. Loglinear models are tested by a goodness-of-fit statistic. $G^2$ models that do not fit the data are rejected by this statistic. Loglinear analysis of 2-dimensional tables is equivalent to the standard $\chi^2$ analysis with the exception that $G^2$ is used rather than the Pearson $\chi^2$ to test for significance. The $G^2$ and $\chi^2$ tests are asymptotically equivalent and yield nearly the same results in moderately large samples.

Sequential loglinear models are a special case in which the form of the model is determined not only by the data but also by a postulated temporal sequence of events. It is important to note that the temporal sequence must be based on prior information, such as previous research or a particular hypothesis. The loglinear analysis can only assess whether the model fits the data. The analysis cannot adjudicate among the various possible sequential models by trying to discover which best fits the data.

**RESULTS**

**Patterns of Oncogene Amplification and Coamplification**

The c-myc oncogene was amplified in 31/84 cases (37%). This exceeded the frequency of HER-2/neu amplification (17/84 cases, or 20%) by nearly two-fold. H-ras was amplified much less often than the others, with a frequency of 8%. When amplified, c-myc alone was amplified in 13/31 cases or 42%. In contrast, when amplified, HER-2/neu and H-ras were each co-amplified with
at least one other oncogene in more than 75% of cases. The relative frequencies of oncogene amplification are consistent with other published studies (+,5,6,9,19,23, 27,33). These patterns of gene amplification suggested that c-myc amplification often occurred early, and was followed by HER-2/neu and ras amplification in individual tumors. JC-A was amplified alone in 35% of cases, and with other oncogenes in 65% of cases. This pattern suggested that JC-A may be amplified early, but it provided no clear indication of the relationship between JC-A amplification and amplification of the other oncogenes studied.

**Loglinear Analysis**

The sample consisted of 61 quadruples of amplification events among four oncogenes: c-myc, JC-A, HER-2/neu, and H-ras, which we designate M, J, H, and R, respectively. Statistical models were designated by a simple coding scheme that lists the components and their proposed associations. For example, (M, J) designates a model consisting of only the c-myc and JC-A oncogenes in which the frequencies of amplification are independent of each other. The model (MJ) specifies the same oncogenes but allows an association to exist between the frequencies of amplification. A more complicated pattern such as (MJ, HR) designates a model encompassing all four oncogenes with one association between c-myc and JC-A and one between HER-2/neu and H-ras, but none of the other four possible two-dimensional associations, none of the four possible 3-dimensional associations, and no 4-dimensional association.

The initial sequential model proposed herein is,

\[ M \rightarrow J \rightarrow H \rightarrow R, \]

also designated (MJ, JH, HR). The model of complete independence among the four oncogenes, (M, J, H, R), was rejected, \( G^2(11) = 52.92, p < .001 \). Thus, some associations among amplification frequencies must exist. We now proceed to build the proposed model component by component.

An association between c-myc and JC-A association, (MJ), exists, as the independence model (MJ) was rejected, \( G^2(1) = 7.20, p < .007 \). Likewise, the partial independence of HER-2/neu from c-myc and JC-A, (MJ, H), was rejected, \( G^2(3) = 12.17, p < .007 \). Unfortunately, the next component of the proposed model, (MJ, JH), was also rejected, \( G^2(2) = 10.25, p < .006 \). Thus, the proposed model cannot account for the observed patterns of amplification frequencies.

The proposed model is rejected, and in principle, we could stop the search for a sequential model of amplification frequencies. However, a slight modification of the proposed model that maintains the sequential ordering is (MJ, JH, MH), which assumes an association between c-myc and HER-2/neu in addition to the JH association. A rival, more parsimonious model is (MJ, MH), which assumes the independence of JC-A and HER-2/neu amplifications. Both the (MJ, JH, MH), \( G^2(1) = 0.97, p < .97 \), and the (MJ, MH), \( G^2(2) = 1.06, p < .59 \), models fit the data, and the latter is chosen on the grounds of parsimony. This gives the sequential model, so far,

\[ J \rightarrow M \rightarrow H \]

as the direction of the arrow between J and M is not a statistical question but a substantive one regarding the biological behavior of oncogenes. We prefer the former sequence.

We continue the model-building process as before, leaving JC-A out of the ordering. The independence of H-ras from c-myc and HER-2/neu, (MH, HR) was rejected, \( G^2(3) = 21.62, p < .0005 \). The model of the conditional association of HER-2/neu and H-ras given the association of c-myc and HER-2/neu, (MH, HR), showed adequate fit to the data, \( G^2(2) = 1.48, p < .45 \). The final model (MJ, MH, HR), displayed as

\[ J \rightarrow M \rightarrow H \rightarrow R \]

showed adequate goodness-of-fit, \( G^2(8) = 4.24, p < .84 \). Table 1, columns 5 and 6 show the observed amplification frequencies and those predicted from this model. The agreement between observed and expected frequencies is good.

**DISCUSSION**

The present study suggests that there is a preferred sequence of gene amplification in human breast cancer. On inspection of the data, it appears that of the mutations investigated, c-myc amplification occurs first. In the present study, c-myc was amplified by more than two-

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Frequencies</th>
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<tbody>
<tr>
<td>c-myc</td>
<td>Observed</td>
</tr>
<tr>
<td>JC-A</td>
<td>29</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>1</td>
</tr>
<tr>
<td>H-ras</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
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<td>3</td>
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<tr>
<td>A</td>
<td>2</td>
</tr>
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\( ^a \text{A = Amplified; U = Unamplified.} \)
fold in 37% of the tumors, the highest frequency observed among the genes that we studied. The frequency of c-myc gene amplification in other published studies has ranged from 1 to 40% (1,4,5,6,17,19,33) with the majority of reported frequencies of amplification in the range of 20-30% (1,4,19,33). Much of the variability in the reported frequencies of c-myc amplification may be due to the admixture of DNA from normal stromal cells and infiltrating leukocytes in varying proportions in different tumors, to differences in patient population characteristics in different studies (1) and to a variety of technical factors that may affect the threshold of detection of gene amplification in different studies. The premise that c-myc amplification occurred first was based on the observation that when c-myc amplification was present, it often occurred alone.

There is some published evidence that supports our finding that c-myc amplification may occur early in the course of breast cancer progression. Watson et al. (33) detected c-myc amplification in ductal carcinoma in situ; c-myc has also been found to be overexpressed in benign fibrocystic disease (28). There is evidence that c-myc overexpression in early breast cancer is due to increased transcription of messenger RNA, and is regulated by estrogen (11,12); in more advanced, hormone-independent cell lines, c-myc overexpression appears to be due to long-lived messenger RNA (11).

The frequency of HER-2/neu gene amplification of 19% reported here is in agreement with results in most other laboratories (4,9,27). In the present study, HER-2/neu was almost always co-amplified with at least one other oncogene (Table 1, row 3, column 3), and when co-amplified, it was most frequently associated with c-myc. The suggestion that HER-2/neu amplification commonly follows c-myc amplification in individual tumors was supported by loglinear analysis. Co-amplification of c-myc and HER-2/neu has been reported in other published studies (1,19).

The overall frequency of amplification of H-ras (8%), and the relatively high frequency of its co-amplification with other oncogenes (Table 1), suggest that it occurred later in a smaller subset of tumors that were more advanced in the genetic evolutionary sequence. The sequence of gene amplification c-myc → HER-2/neu → H-ras was supported by loglinear analysis.

It is apparent that amplification of other as yet uncharacterized genes contributes to breast cancer progression. Comparative genomic hybridization (15) and cytogenetic techniques (35) have revealed gene amplification in chromosomal regions that do not correspond to the loci of known oncogenes. It would be worthwhile to identify some of these genes and correlate their presence with clinical outcome. JCA is an anonymous amplified gene fragment isolated in our laboratory from a tumor obtained from a patient with advanced disease. This fragment has tentatively been localized to chromosome 19 (unpublished observations) and was able to detect moderate increases in copy number in 17 of 61 breast cancers examined (Table 1). When these data were analyzed statistically, JCA amplification either followed or preceded that of c-myc in the genetic evolutionary sequence. Its amplification, however, was unrelated to that of HER-2/neu and H-ras. More detailed analysis awaits further characterization of JCA in relation to clinical parameters and the presence of other genetic alterations in breast cancer.

The molecular genetic techniques we have used cannot distinguish among the various genetic mechanisms that can lead to increased gene copy number per cell, such as the production of intrachromosomal tandem gene reduplication (29), the production of double minutes (20), and the selective retention of multiple copies of oncogene-bearing chromosomes (34) during the course of repeated rounds of chromosome complement doubling with chromosome loss. Indeed, it is quite possible that the relatively infrequent, apparent gene amplifications of H-ras that we observed, may actually be due to the selective retention of multiple copies of chromosome 11 in aneuploid cells. It may also occur randomly as a consequence of genetic instability in solid tumors in advanced stages of progression.

The distinctions among the various mechanisms responsible for gene amplification can be made by means of fluorescence in situ hybridization studies using fluorescent probes for specific chromosomes and for specific genes in the same cells. Such two-color studies by Kallioniemi et al. (15), have demonstrated increased HER-2/neu gene copy number at several chromosomal loci in the same breast cancer cells in some tumors. The data of these authors suggest that the effects of tandem gene reduplication can be leveraged by chromosome complement doubling in the same cell to produce overall oncogene copy number as high as 50-75 per cell (15).

In summary, our studies suggest that there is a preferred sequence of oncogene amplification and overexpression in breast cancer. Future strategies for optimizing combinations of prognostic factors might focus on the interrelationships among these factors.

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