Amplification of c-myc but not of c-erbB-2 Is Associated with High Proliferative Capacity in Breast Cancer

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ABSTRACT

Proliferative capacity provides an independent prognostic marker of progression in breast cancer. Little is known about the molecular mechanisms influencing the cell division rate in mammary carcinomas. In order to address this issue, the copy numbers of c-erbB-2 (HER/neu) and c-myc protooncogenes that have been shown to be amplified in aggressive types of cancers were determined in 60 mammary carcinomas and related to the proliferation rate. The proliferative activity was determined by labeling of the proliferation-associated nuclear antigen which is defined by the recently described monoclonal antibody Ki-S1. Approximately one-third of samples under investigation displayed a Ki-S1 labeling index exceeding 30%. In this subgroup, amplification of c-myc was found in 52.6%, whereas in the remaining cases, 26.1% exhibited an enhanced copy number of c-myc (P < 0.025). By contrast, c-erbB-2 amplification was not found to be associated with a higher proliferation index. Except for one case of invasive lobular carcinoma, both protooncogenes exhibited regular copy numbers in the low proliferation subgroup (<20%; P < 0.03). We conclude from our findings that c-myc amplification may be one of the molecular causes underlying the highly proliferating phenotype of mammary carcinoma, known to be associated with an unfavorable clinical course.

INTRODUCTION

There is a growing body of evidence indicating that the progression of breast cancer is determined to a considerable extent by the proliferative activity of tumor cells (1). This has been demonstrated by different methods suitable for the assessment of the tumor cell growth fraction (2–5). Recently, we could show that the immunohistochemical detection of the proliferation-associated nuclear Ki-S1 antigen (6) provided a reliable instrument to determine the growth fraction of mammary carcinomas, yielding a highly significant correlation with the S-phase fraction and the clinical outcome (7, 8). The expression of the Ki-S1 antigen parallels that of the established marker of cell proliferation Ki-67 (9), being positive from G1 to G2/M and negative in G0 (6). Unlike Ki-67 the epitope binding Ki-S1 is resistant to formalin fixation and paraffin embedding, enabling retrospective analysis of archival tissue samples with clinical follow-up of 12 years and more (8).

Little is known about the molecular mechanisms governing the recruitment of breast cancer cells into the cell cycle. One potential cause might be provided by deregulated expression of c-myc that represents an immediate early response gene activated rapidly after growth factor-induced commitment to enter the cell cycle (10). A number of studies have demonstrated a positive correlation between amplification, a frequent mechanism involved in the activation of c-myc to a transforming oncogene, and the biological behavior of mammary tumors. However, the proportion of amplification positive tumors varied among different studies (1–41%), and a positive correlation with prognostic covariates could not constantly be established (11–15). Using an immunohistochemical approach Pavelic et al. (16) could demonstrate a correlation between c-myc and Ki-67 labeling indices. From this phenotypic analysis it could not be decided whether the enhanced c-myc expression was causative for the increased proliferative activity because both features might have been induced by an independent regulatory defect.

Another protooncogene implicated in the pathogenesis of aggressive breast cancer is the c-erbB-2 (HER/neu) gene coding for an epidermal growth factor-related receptor (17, 18) with an as yet incompletely characterized ligand (19–21). Amplification of the gene (22) and overexpression (23) have been described in aggressive types of breast cancer. These observations were not confirmed by all investigators, some of them finding a significant correlation with prognosis to be confined to particular histological or clinicopathological subtypes (24). Many of these studies used immunohistochemical methods to detect overexpression in fixed tissues (24–27). A limiting factor to this approach is provided by the inconstant preservation of antigens in paraffin-embedded tissues causing varying thresholds of detection. By contrast, the determination of DNA amplification as originally described by Slamon et al. (22) appears to be more reproducible and appropriate for standardization.

In order to get a further insight into the genetic aberrations determining the cycling rate of breast cancers we have investigated the amplification state of c-myc and c-erbB-2 protooncogenes in mammary tumors and related it to the proliferative index, whereby a significant correlation between c-myc amplification and the magnitude of the growth fraction could be shown.

MATERIALS AND METHODS

Tissue Specimens and Cells. Primary mammary carcinomas from 60 patients were included in the study comprising the following histological subtypes: 56 invasive ductal carcinomas; 2 invasive lobular carcinomas; one intraductal carcinoma, comedo type; and 1 mucinous carcinoma; histological subtyping and grading were performed according to established criteria and recommendations (28). From each specimen adjacent tumor-free tissue was taken for analysis. The specimens were divided into three probes that were used for routine histopathological consideration and as unfixed fresh tissue for immunohistochemistry and isolation of DNA. The latter probes were stored immediately at −80°C after snap freezing in liquid nitrogen.

In addition, the breast carcinoma-derived cell line MCF7 (obtained from the American Type Culture Collection) (29) was kept under standard culture conditions and used for the extraction of DNA. Furthermore, blood granulocytes from healthy donors (n = 3), isolated as described (30), served as a source of control DNA.

Immunohistochemistry. Immunohistochemical staining of frozen sections was performed as described with minor modifications using biotin streptavidin and peroxidase for visualization (6). From paraffin-embedded tissues 3–5-μm sections were prepared and deparaffinized in xylol, rehydrated, and incubated for 5 min with 3% hydrogen peroxide in methanol. The Ki-S1 antigen was unmasked by treatment with 0.1% trypsin (Sigma, Steinheim, Germany) for 45 min at 37°C. After rinsing, Ki-S1 antibody concentrated to 15 μg/ml and diluted 1:60,000–1:100,000 or culture supernatant diluted up to 1:100 was added and incubated for 30 min. Visualization was performed with the streptavidin-biotin complex using peroxidase and rabbit-antimouse antibody (all Dako, Hamburg, Germany) (6). The immunohistochemical detection and analysis of steroid hormone receptors were carried out as described (31).
Evaluation of the Proliferation Index. Three to five areas in a paraffin section exhibiting intense nuclear staining and low background staining were selected. Within these areas 500 infiltrative, growing tumor cells were counted, and the percentage of tumor cells with positive nuclear staining was registered. In the case of the noninfiltrating tumor, intraductal areas with the highest density of positively labeled nuclei were chosen for analysis. At least 2 sections were cut from each sample and stained with 2 different dilutions of the antibody (1:60,000 and 1:100,000) or with Ki-S1 culture supernatant diluted by 1:100. Questionable cases were restained or excluded from the study. False positive staining was indicated by intense nuclear staining of nonproliferating stromal cells. False negative staining was presumed when mitotic figures displayed negativity.

DNA Analysis. DNA was isolated from fresh stored tissue samples as described (32) using a lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, 2% sodium dodecyl sulfate) and proteinase K (1 µg/ml; Merck, Darmstadt, Germany) followed by extraction with phenol/chloroform. DNA was precipitated by the addition of ethanol and resolved in water. The DNA concentration was determined with a fluorimeter (Kontron, Munich, Germany) using yeast DNA as standard. Restriction of DNA was performed with EcoRI according to the recommendations of the supplier (Boehringer Mannheim, Mannheim, Germany). Fifteen µg of cut DNA were subjected to electrophoretic separation in 1% agarose gels and transferred to nylon membranes (Genescreen; Dupont, Dreieich, Germany). Prehybridization and hybridization of filter membranes, radioactive labeling of DNA probes with 32P by the oligolabeling method (33), and autoradiography were carried out as described (30). The following DNA probes were used for hybridization: a 1.3-kilobase EcoRI/ClaI fragment subcloned from a 9.0-kilobase EcoRI/HindIII fragment encompassing human c-myc exon 3, obtained from Oncogene Science (Manhasset, NY) (34); a 300-base pair fragment of the erbB-2 promoter generated by polymerase chain reaction according to the published sequence using a CAGAGTCACCAGCCTCTGCAT and a CTGGTTTCTCCGGTCCCAATG oligonucleotide as primers (35); and a 2.0-kilobase BamHI/HindIII complementary DNA fragment of the human γ-interferon receptor, obtained as a generous gift from Dr. Z. Dembic (Basel, Switzerland) (36). Hybridization lasted 48 h at 42°C. All three labeled DNA probes were contained simultaneously in the hybridization buffer. Washings were carried out at 60°C with 1× standard saline citrate (32) for 30 min. Exposure of autoradiography film lasted between 12 h and 1 week.

The signals obtained were evaluated densitometrically using a Bio Profile device (Vilber Lourmat, Marne La Vallée, France). Densitometric values were expressed as multiples of the control signal (interferon receptor). Two types of control cells were used in order to define the ratio of the hybridization signals in normal tissues. Because the possibility could not be excluded that apparently normal tissue adjacent to carcinoma samples contained precursor lesions with already altered gene copy numbers, normal blood granulocytes, in addition to mammary tissue, served as sources for control DNA. However, no differences between these two types of control DNA were found. In control samples, the mean ratios of c-myc to control gene and c-erbB-2 to control gene were 0.87 and 0.28, respectively. The hybridization conditions were modulated accordingly to generate relatively weaker signals of the protooncogenes for easier detection and quantification of amplification. Tumors were considered to display amplification of c-myc or c-erbB-2, respectively, when the relative intensity of the corresponding autoradiographic signal exceeded that observable in normal cells by a factor of ≥1.8. This value was chosen for two reasons. First, in the control specimens that were run in each hybridization the relative intensity of both protooncogene signals proved to be within narrow borders, never exceeding a factor higher than 1.3. Second, because of contaminating stromal cells and infiltrating leukocytes in tumor specimens amplification could be presumed even with relative values lower than a factor of 2.

Statistical Analysis. The effect of protooncogene amplification on proliferative capacity and other prognostic covariates was examined by a standard univariate χ² test. A P value of 0.05 or less was considered statistically significant.

RESULTS

Proliferative Capacity of Breast Cancers. Nuclear staining of tumor cells with the monoclonal antibody Ki-S1 ranged from 8% to 70%, with a median of 25%. When heterogeneous staining patterns occurred within a given tumor, areas with the highest labeling intensity were considered for evaluation of the proliferation index. Examples of tumors with a low, medium, and high proliferation rate as determined by nuclear expression of the Ki-S1 antigen are shown in Fig. 1. Some tumors displayed low proliferation indices not exceeding 10% (Fig. 1a). As can be seen from the histological appearance of the tumors shown in Fig. 1 a low proliferation index was usually associated with a higher degree of differentiation and a lower histological grade of malignancy. However, some tumors with a high nuclear and histological grading exhibited Ki-S1 indices below 20%. Most tumors belonged to the group with intermediate proliferative capacity which
exhibited Ki-S1 labeling of 20% to 30% of tumor cell nuclei (Fig. 1b).
Nineteen breast cancer samples (31%) showed nuclear labeling by
Ki-S1 in more than 30% of cells, and in some cases the rate of labeling
rose to 70% (Fig. 1c). Since in a previous study breast cancers with a
Ki-S1 labeling index exceeding 30% had revealed a significantly
worse clinical outcome (7) this value was chosen as the cutoff level.
In the high-proliferation subgroup (≥30%) 84% of tumors belonged to
the grade 3 group, whereas in the low-proliferation subgroup (<20%)
38% were thus categorized.

Amplification of c-myc and c-erbB-2 in Breast Cancers. Southern
blot analyses of c-erbB-2, c-myc, and the gene for the interferon
receptor in granulocytes and normal mammary tissue adjacent to
tumors are shown in Fig. 2. Granulocytic DNA (Fig. 2, Lane 1) and
DNA from normal mammary tissue (Fig. 2, Lanes 2 and 3) rendered
identical ratios of the intensities of the hybridization signals. The
mammary carcinoma-derived MCF7 cell line revealed amplified copy
numbers of c-myc (Fig. 2, Lane 4). Representative examples of tumors
exhibiting amplified copy numbers of c-myc and c-erbB-2 are shown
in Figs. 3 and 4, respectively. Aberrant fragments were not noticed
in any case. Of the 60 tumors under investigation 20 (33%) displayed
amplification of c-myc, and 16 (26.6%) cases exhibited amplified
copy numbers of c-erbB-2.

The amplification of c-myc ranged from 1.8 to 8, and the amplifi-
cation of c-erbB-2 ranged from 1.8 to 19. There was a tendency in
c-myc amplified tumors toward enhanced lymph node involvement
and larger tumor size; however, these did not reach the level of
significance (Table 1). There was no significant correlation between
either c-myc or c-erbB-2 amplification and the age, hormone recep-
tor status, or histological grade (Tables 1 and 2). A weak correlation
was found between histological grade and c-erbB-2 amplification
(P < 0.1) that also did not reach the level of significance. In addition,
no significant correlation could be shown between increased c-erbB-2
copy numbers and tumor size or lymph node status.

Correlation of Proliferative Capacity and Protooncogene Am-
plification in Breast Cancers. When the results of Ki-S1 staining
were compared with the DNA analysis a weak but significant corre-
lation could be demonstrated between c-myc amplification and prol-
eration index (P < 0.025; x² = 6.23). By contrast, c-erbB-2 was not
found to be significantly correlated to proliferative activity. This also
held true when only tumors with an amplification rate exceeding 5
(n = 4) were taken into consideration. Conversely, when the low-
proliferation tumors with a proliferation index of <20 (n = 13) were
analyzed for protooncogene amplification, it turned out that low Ki-S1
expressing tumors exhibited amplified c-myc or c-erbB-2 only occa-
sionally. Only one case of infiltrating lobular carcinoma with a com-
parably low proliferation rate displayed c-myc protooncogene ampli-
fication, whereas none of these tumors revealed an enhanced copy
number of c-erbB-2. The correlation was found to be significant with
regard to both protooncogenes (P < 0.03, x² = 5.14 and 4.84,
respectively) (Tables 1 and 2).

DISCUSSION

Little is known about the molecular defects that distinguish breast
cancer with high proliferative capacity from the majority of mammary
tumors. It has been suggested that p53, which probably plays a crucial
role in cell cycle control (37), is more frequently affected by mutations
in the highly proliferating subgroup (38, 39). Probably there is another
gene near p53 at chromosome 17p, which by loss of function can give
rise to the rapidly cycling phenotype (40-42).

The c-myc protooncogene has been implicated in the pathogenesis
of aggressively growing mammary tumors (11-15). Despite its clear
association with fundamental normal and aberrant cellular processes,
such as growth factor signaling and DNA replication, the direct bio-
chemical activities of c-myc protein still remain elusive (10). Dereg-
rulation of c-myc has been shown to prevent differentiation (43), and
mice transgenic for c-myc develop a variety of tumors including those
of breast (44).
A frequent mechanism leading to the deregulation of c-myc expression is gene amplification (10). In addition to structural defects by insertional mutagenesis (45), it is the amplification of c-myc which has been most described in breast cancer (11–15, 46–49). The frequency of amplified cases ranged from 1% (47) to 41% (12). The reasons for these differences are not evident. Some of these studies considered 3 or more copies as amplification (15); others set the threshold at 2 copies (11–14). Escot et al. (11) described a 2–15-fold c-myc amplification in 32% of tumors and stated that this magnitude and frequency was probably an underestimate due to the variability of the tumor tissue:stroma ratio and the potential presence of normal breast tissue in the samples under investigation. The magnitude and frequency of c-myc amplification as assessed in our investigation are well in line with those described in most studies (11–15, 46–49). A significant correlation with a worse clinical outcome was reported in some (15, 48) but not all studies. Also, the association with the lymph node status is controversial (11, 13, 46, 49). In our study there was a trend toward a higher frequency of lymph node involvement in c-myc amplified cases that was, however, not statistically significant. The association of c-myc amplification with a higher proliferation index points to a more aggressive behavior of tumors with enhanced c-myc copy numbers. Moreover, the established functional activities of the myc protein are consistent with a deregulation of proliferation in tumors bearing a c-myc gene amplification. In a large series of breast cancers comprising 282 cases Berns et al. (15) demonstrated c-myc amplification to be a reliable prognostic indicator.

In addition to c-myc, c-erbB-2 (HER.2/neu) has been implicated in the pathogenesis of breast cancer. Coding for an epidermal growth factor receptor-related protein (17, 18) the erbB-2 gene may be activated to a transforming oncogene by point mutations (50, 51). Transgenic mice bearing the activated oncogene uniformly develop mammary adenocarcinoma (52). In human tumors, however, point mutations of c-erbB-2 are a rare event (23), whereas amplification does frequently occur, ranging from 9% to 30% of cases (22, 47). Amplification is almost constantly associated with enhanced expression of the transcript or protein (23). In nodal positive cases c-erbB-2 amplification is associated with an unfavorable clinical course (22). However, the value of c-erbB-2 amplification as a prognostic indicator is the subject of controversy (53). Few studies have focused on the influence of c-erbB-2 on the proliferative behavior of tumors. Two investigations described an increased S-phase fraction in amplified or overexpressing cases (27, 54). Except for an absence of c-erbB-2 amplification in low proliferating tumors (Table 2) we did not find a significant correlation between amplification and heightened proliferation index. Similarly, Pavelic et al. (16) did not find a relationship between c-erbB-2 overexpression and Ki-67 index. It remains to be clarified why c-erbB-2-amplified tumor cell clones are preferentially selected for outgrowth if they do not experience a proliferative advantage from this genomic alteration. A positive correlation between c-erbB-2 amplification and a high rate of proliferation has been described by Borg et al. (55). However, the correlation was confined to steroid receptor-positive breast cancers and was not found in receptor-negative tumors (55).

The amplification rates of c-myc and c-erbB-2 detected in this study are well in line with those reported in the literature. Therefore, the simultaneous hybridization procedure as performed in this study ap-

### Table 1 Correlation of c-myc amplification with prognostic covariates in mammary carcinomas

<table>
<thead>
<tr>
<th>Covariate or category</th>
<th>Germ line (n = 40)</th>
<th>Amplified (n = 20)</th>
<th>Correlation</th>
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<tbody>
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<td>Age (years)</td>
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<td></td>
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<tr>
<td>≤49</td>
<td>10</td>
<td>2</td>
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<tr>
<td>50–74</td>
<td>23</td>
<td>16</td>
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<td>≥75</td>
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<td>2</td>
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<tr>
<td>Tumor size (mm)</td>
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<tr>
<td>&lt;20</td>
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<tr>
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<td>18</td>
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<tr>
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<td>6</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
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<td>5</td>
</tr>
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<td></td>
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<td></td>
<td>N2</td>
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<tr>
<td></td>
<td>NX</td>
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</tr>
<tr>
<td>Histological grade&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>9</td>
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<tr>
<td></td>
<td>III</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>Ki-S1 index&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;20%</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>&lt;30%</td>
<td>31</td>
<td>10</td>
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<sup>a</sup> Hormone receptor status was not available for all samples. The receptor status was determined immunohistochemically.

<sup>b</sup> Lymph node involvement was categorized according to the tumor-node-metastasis scheme of the International Union Against Cancer: metastasis without (N1) and with (N2) perinodal infiltration and fixation. NX, unknown nodular status.

<sup>c</sup> Grading was performed only on invasive ductal carcinomas (n = 56) according to Ref. 28.

<sup>d</sup> Percentage of carcinoma cells exhibiting positive nuclear staining with the monoclonal antibody Ki-S1.

### Table 2 Correlation of c-erbB-2 amplification with prognostic covariates in mammary carcinomas

<table>
<thead>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
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<tr>
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<sup>c</sup> Grading was performed only on invasive ductal carcinomas (n = 56) according to Ref. 28.

<sup>d</sup> Percentage of carcinoma cells exhibiting positive nuclear staining with the monoclonal antibody Ki-S1.
pears to represent an easy and reliable method for the assessment of protooncogene amplification in clinical specimens. The control gene probe has been chosen because of the appropriate fragment size in the EcoRI digest and its localization to a chromosomal region only infrequently involved in defects in mammary carcinomas. There have been reports on 6q deletions in breast cancers, but the breakpoints were located distal to the chromosomal localization of the \( \gamma \)-interferon receptor gene (56, 57). Because the observed frequencies of c-myc and c-erbB-2 amplification are in accordance with those reported in the literature and because no correlation between amplification of c-myc and c-erbB-2 could be established, it seems very unlikely that a loss of the chromosomal region harboring the control gene mimicked increased copy numbers of protooncogenes in the cases under investigation.

In conclusion, we have provided evidence for an involvement of c-myc amplification in the pathogenesis of the highly proliferating phenotype of breast cancer. Besides c-myc amplification there are probably different molecular defects that enable tumor cells to proliferate more rapidly. Their elucidation might represent an important step toward the identification of a high-risk subgroup among breast cancer patients.

REFERENCES


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