Gelsolin as a Negative Prognostic Factor and Effector of Motility in erbB-2-positive Epidermal Growth Factor Receptor-positive Breast Cancers

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INTRODUCTION

Clinical and pathological markers (such as nodal status, size, stage, grade, and so forth) provide important prognostic information for breast cancer patients. More recently, molecular markers, including erbB-2, have been reported with prognostic or predictive value. Interactions between erbB-2 and other related receptors, such as the EGFR, are well known, although linkage between downstream effects and patient outcome are less well understood.

erbB-2 is a Mr 185,000 cell surface receptor (1), which is a member of the tyrosine receptor kinase superfamily. erbB-2 is overexpressed and the gene is amplified in approximately one-third of breast cancers (2). erbB-2 overexpression has been associated with a poor prognosis, particularly for patients with node-positive disease. erbB-2 activation may occur via several mechanisms; this often involves heterodimerization with other family members, such as EGFR.

EGFR is a Mr 170,000 transmembrane receptor protein that binds to EGF, tumor necrosis factor, and other ligands (3, 4). Up-regulation of EGFR increases cellular sensitivity to both autocrine and paracrine growth stimulation (5–8). EGFR may be induced by estrogen via direct binding to the promoter region (9–11). Ligand binding to EGFR facilitates heterodimer formation with erbB-2 and triggers a rapid tyrosine phosphorylation of the erbB-2 protein as well as other downstream substrates (12, 13). Overexpression of EGFR, generally in the absence of gene amplification, has been reported in a wide range of breast cancers (generally one-fifth to one-half of cases). EGFR overexpression has been associated with anchorage-independent growth (14), increased metastatic potential, and a worse prognosis in both node-positive and node-negative breast cancer patients (15–21).

Gelsolin is widely expressed by normal cells (22) and may be down-regulated with transformation of multiple cell types, including breast epithelium (23, 24). It has therefore been considered a candidate tumor suppressor gene (Refs. 23, 25, and 26; data not shown). Although gelsolin-null mice survive, they have multiple defects in cell morphology and motility, including neutrophils, platelets, osteoclasts, and dermal fibroblasts (27, 28), as well as a delay in breast development (29). Gelsolin expression is not detectable in many breast cancers, although more than one-third of cases may have detectable expression of gelsolin RNA or protein (23, 24). High gelsolin levels have recently been identified as a negative prognostic factor in pul-

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The abbreviations used are: EGFR, epidermal growth factor receptor; erbB, erbB family; EGF, epidermal growth factor; PLC, phospholipase C; ER, estrogen receptor; MI, mitotic index; DFS, disease-free survival; DSS, disease-specific overall survival; Gt, tumor gelsolin; Gs, stromal gelsolin.

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monary carcinomas [stage I non-small cell lung carcinomas (30)], where they have been linked to enhanced cellular motility.

Gelsolin is a M, 80,000 actin-binding protein that participates in and regulates dynamic changes in the actin cytoskeleton. The actin cytoskeleton produces the physical force necessary for cellular integrity and enables diverse forms of cell motility. Gelsolin binds to the side of actin filaments (in micromolar concentrations of Ca\(^{2+}\)) and cuts them into smaller pieces. Gelsolin then can specific sites of actin filaments at the rapidly growing end. Gelsolin dissociates from the ends of actin filaments by lowered Ca\(^{2+}\) concentrations and contact with phosphoinositides of the D4 type, providing sites for rapid actin filament extension. EGFR/erbB-2 activates PLC\(\gamma\), which activates a motility pathway that is dependent on gelsolin expression to induce modification of the actin cytoskeleton (31). This interaction between erbB-2/EGFR and gelsolin activation has been called the “master switch” hypothesis (32).

Mechanisms by which erbB-2 and EGFR expression confer a worse clinical outcome for breast cancer patients are not well understood. In this study, we explore the master switch hypothesis by examining expression data for erbB-2, EGFR, and gelsolin in nearly 800 archival breast cancers. Statistical associations between these data and clinical, pathological, and outcome information are presented.

PATIENTS AND METHODS

Patient Population. A total of 790 archival formalin-fixed paraffin-embedded breast cancers from patients treated at the Massachusetts General Hospital (Boston, MA) between 1976 and 1983 were available for correlative study. Tumor size ranged from 0.2–16 cm, with a mean tumor size of 2.9 cm. Patient age ranged from 24–95 years, with a mean age of 60 years. Lymph node status was determined by nodal dissection and histological examination in 678 (86%) patients; 336 (50%) were node negative, and 342 were (50%) node positive. Follow-up was calculated from the date of surgery to the last recorded follow-up date (mean follow-up, 15.6 years; median follow-up, 16.3 years). Local recurrence and distant metastasis were calculated from the date of surgery to the first documented failure date.

Tumors were regraded using the Nottingham combined histological grading scheme (33). This resulted in 70 (9%) grade 1 tumors, 356 (47%) grade 2 tumors, and 332 (44%) grade 3 tumors. Thirty-four tumors (4%) were classified as lobular carcinoma according to the WHO schema (34) and were not graded (in general, invasive ductal cancers were favored for selection in this bank). ER data determined by either the initially applied charcoal dextran assay or the more recently developed immunohistochemical assays (35, 36) resulted in 347 (44%) ER-negative cases, 440 (56%) ER-positive cases, and 5 cases with unknown ER status (<1%). The MI was determined by counting the number of mitotic figures/10 high-powered microscopic fields, as described previously (37). The median MI for all cases was 6 mitoses/10 high-powered microscopic fields. MI-1-derived proliferation data were available on 777 of these breast cancers. MI-1 data ranged from 0–95.7% of invasive cancer cells positive, with a median of 17.8% for all cases.

Immunohistochemical Staining. Slides with primary, invasive breast cancer were stained with monoclonal anti-erbB-2 (CB11; BioGenex, San Ramon, CA; dilution, 1:900) using previously described methods (2, 38). We have recently reported comparability of this reagent and method with other immunohistochemical anti-erbB-2 reagents and two fluorescence in situ hybridization methods kits (39). In addition, we have shown that erbB-2 data derived using this immunohistochemical reagent and method is highly correlated with independently derived fluorescence in situ hybridization or differential PCR-based molecular methods to evaluate erbB-2 abnormalities (2).

EGFR staining was performed using clone 1005 polyclonal anti-EGFR antibody (dilution, 1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; data not reported previously). A similar staining pattern was also observed using monoclonal antibody E30 (1:200 dilution) in a parallel study of 40 selected cases (data not shown). In these 40 cases, distinct subsets of cancers were identified that were EGFR+ and erbB-2− or vice versa, hence we have no evidence of cross-reactivity between anti-EGFR and anti-erbB-2 reagents. Both erbB-2- and EGFR-stained tumor slides were evaluated in toto for membranous immunohistochemical positivity of invasive tumor cells. For each, an estimate of the percentage of positive cells (0–100%, continuous data) was recorded. These continuous data were used for all statistical analyses with the exception of the univariate odds ratios and Kaplan-Meier survival curves, which required dichotomization (see below).

Cellular gelsolin was also identified by immunohistochemical methods, using an overnight incubation with monoclonal anti-gelsolin (2C4; dilution, 1:200; provided by D. J. K.) at 4°C. Antibody visualization and interpretation were similar to those described above, except that a cytoplasmic expression pattern with membranous accentuation was generally observed. This expression was focal in some cells (see “Results”). Cytoplasmic gelsolin was also observed in tumor cells and in intratumoral and peritumoral stromal fibroblasts (as reported by others). The entire slide was examined for gelsolin positivity. Separate scores were generated for invasive tumor cells or stromal fibroblasts; estimates of the percentage of these cells positive for gelsolin were recorded.

Other immunohistochemical expression data available for these tumors included p53 [PAb 1801; Genesis Bio-Pharmaceuticals, Inc., Tenafly NJ; dilution, 1:4000 (40)] and the proliferation marker MIB-1 [AMAC, Inc., Westbrook, ME; 1:200 dilution after antigen retrieval (37)]. For each of the above-mentioned assays, fixed embedded positive control and negative control cell lines were used to ensure interassay reproducibility and specificity.

Statistical Methods. Pearson product moment correlations were used to describe the associations between erbB-2, EGFR, and gelsolin expressed as continuous variables (0–100%) and compared with clinical, pathological, and immunohistochemical variables. Unpaired t tests were used to describe the associations between erbB-2, EGFR, and gelsolin expression as continuous variables (0–100%) and lymph node status. For univariate survival analyses, factors were separated by cut points into two or three groups, and odds ratios and confidence levels were calculated. The log-rank statistic was
used to determine significance. Survival analyses compared the
prognostic value of erbB-2, EGFR, and gelsolin overexpression
as either individual or clustered markers. The outcomes selected
for study included DFS (defined as time to local recurrence or
distant metastasis) and DSS (defined as time to death from
breast cancer). Patients who died of causes unrelated to breast
cancer were censored at date of death. Patients who died of
unknown causes were excluded from disease-specific survival
analyses. The Cox proportional hazards model was used to
calculate both univariate and multivariate survival (41).

For multivariate survival, we first constructed models that
included all factors univariately associated with survival (ex-
cluding erbB-2, EGFR, and gelsolin). We then removed the
variables one at a time, based on the Wald statistic, until only
statistically significant variables remained. Each of the variables
of interest (erbB-2, EGFR, and gelsolin) was added individually
to the models as either a continuous or dichotomized variable
using a cut point of 0 versus ≥1% immunostaining. New com-
posite variables were also constructed to test interactions be-
tween erbB-2, EGFR, and gelsolin. To test the two-way in-
teraction between erbB-2 and EGFR, the variable developed
(erbB-2 × EGFR) included the following elements: erbB-2–
EGFR−; erbB-2– EGFR+; erbB-2+ EGFR−; and erbB-2+ EGFR+.
This resulted in an interaction term with 3 degrees of
freedom. Then, to examine the three-way interaction between
erbB-2, EGFR, and gelsolin, a tricomponent variable was con-
structed (erbB-2 × EGFR × gelsolin). erbB-2 × EGFR ×
gelsolin had 6 degrees of freedom because there were no pa-
tients in the erbB-2– EGFR+ gelsolin− group. The prognostic
results using the variable erbB-2 × EGFR × gelsolin were then
compared with the results using the variables erbB-2 × EGFR
and gelsolin for each model.

The number of positive lymph nodes was logarithmically
transformed (after adding the constant 1 to avoid taking log of
0 for node-negative cases) because a plot of Martingale resid-
uals against number of positive nodes suggested that this transfor-
mation gave a better linear relationship between risk and
nodes. Tumor size, grade, age, MI, and the proliferation marker
MIB-1 were entered as continuous variables, and biochemical
ER was dichotomized with a cut point of 10 fmol/mg protein or
≥20% of tumor cells positive by immunohistochemistry (in-
stitutional cut point). p53 was entered into the statistical models
as a binary variable ≥1, where any staining was considered posi-
tive, and no staining was considered negative.

RESULTS

Clinical, Histological, and Marker Data. A total of 790
primary invasive breast cancers were studied for erbB-2, EGFR,
and gelsolin expression. Each also had other clinical, patholog-
ical, and immunohistochemical data. erbB-2 positivity was ob-
erved in 303 (38%) of breast cancers (range, 0.5–100% of cells
were erbB-2+); of these, 222 showed ≥10% positivity (a cut
point commonly used in HER2 testing). EGFR staining was
present in 122 breast cancers (15%; range, 0.5–99%). Gt was
cytoplasmic with membranous accentuation and was observed
in 443 cases (56%). Gelsolin expression was also observed in
stromal fibroblasts (Gs) in 630 (80%) patients.

erbB-2 expression was seen in 130 of 336 (39%) node-
negative patients and in 140 of 342 (41%) node-positive pa-
tients. Using a cut point of 10%, 89 node-negative and 108
node-positive patients would be considered overexpressors. For
erbB-2 staining, a bimodal distribution of data was observed,
with many cases (72%) exhibiting no or <10% reactivity and
another group of cases (20%) having >40% reactive invasive
tumor cells. EGFR expression was observed in 35 (10%) node-
and 73 (21%) node-positive cancers. Gt positivity was
observed in 172 (51%) node-negative tumors and 214 (63%)
ode-positive tumors.

Associations between erbB-2, EGFR, Gt, and other clinical
and histological features of these patients are shown in Table 1.
As shown in Table 2, both erbB-2 expression and EGFR
expression were also correlated with other poor prognostic
markers. Gt, erbB-2, and EGFR were positively correlated with
nodal metastases if nodal status was considered as a dichoto-

mous (negative or positive) variable or a three-way variable
(negative, 1–3, and 4+) using the χ² statistic. None of the three
factors (erbB-2, EGFR, or Gt) was associated with the number
of positive nodes (Table 1). Gt was associated with young
patient age, high tumor grade, high MI, negative ER, and
positive staining for erbB-2, EGFR, p53, and proliferation rate
(MIB-1).

In the lymph node-negative subset, erbB-2, EGFR, and Gt
were also correlated with each other (P < 0.003 for all). Gt was
also positively correlated with tumor grade, p53 immuno-
staining, and proliferation and negatively correlated with ER
status in the lymph node-negative subset. Among the lymph
node-positive subset, erbB-2, EGFR, and Gt were similarly
associated. Associations with other factors were similar to those
observed for all patients (data not shown).

Univariate Analysis of Survival. As shown in Table 2,
many factors, including erbB-2 and EGFR, were significantly
associated with DFS and DSS for all patients. Neither Gt nor Gs
was correlated with DFS or DSS when examined in isolation.
For lymph node-negative patients, none of the three factors
(erbB-2, EGFR, or Gt) was associated with either DFS or DSS
(n = 326; data not shown). Factors associated with DFS and
DSS in the lymph node-negative patients included tumor grade
and cellular proliferation (MIB-1 and MI). Factors significantly
associated with DFS alone in these patients included patient age,
tumor size, and p53 immunopositivity (data not shown).

In the node-positive patient subset (n = 336), both erbB-2
and EGFR were highly correlated with both DFS and DSS (P ≤0.0002 for each). Neither Gt nor Gs alone was associated with
outcome (DFS or DSS). Other factors associated with DFS and
DSS for node-positive patients included tumor grade, lymph
node positivity, ER status, and MIB-1 greater than the median
value. MI and p53 expression were associated with DSS but not
with DFS (data not shown).

Multivariate Survival Analysis. Multivariate analyses
to identify base measures independently associated with DFS
were performed. This included all variables except those to be
tested (erbB-2, EGFR, and Gt; Table 3). The number of positive
lymph nodes, histological tumor grade, tumor size, and ER
status were all significant factors for DFS. Both erbB-2 (as a
continuous variable) and EGFR (as a continuous variable or a
dichotomous variable) added significantly to this base model or
Table 1. Pearson product moment correlations between erbB-2, EGFR, gelsolin, and other variables for all patients

<table>
<thead>
<tr>
<th>Factor</th>
<th>Correlation Coefficient (r)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.076</td>
<td>-0.16 to -0.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>Size</td>
<td>0.208</td>
<td>0.13 to 0.27</td>
<td>0.0001</td>
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<tr>
<td>ER status</td>
<td>0.306</td>
<td>0.22 to 0.38</td>
<td>0.0001</td>
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<tr>
<td>ERP</td>
<td>0.256</td>
<td>0.17 to 0.32</td>
<td>0.0001</td>
</tr>
<tr>
<td>MB-1</td>
<td>0.182</td>
<td>0.11 to 0.25</td>
<td>0.0001</td>
</tr>
<tr>
<td>MB-2</td>
<td>0.129</td>
<td>0.06 to 0.21</td>
<td>0.0145</td>
</tr>
<tr>
<td>Gt</td>
<td>0.138</td>
<td>0.07 to 0.21</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

DFS (Table 3). Gt (as either a continuous or dichotomous factor) was not significantly associated with survival prediction for DFS among all patients (Table 3). The addition of erbB-2 to the DFS model eliminated the significance of tumor grade. The addition of EGFR expression as a continuous variable reduced the significance of tumor grade. The addition of EGFR as a dichotomous variable reduced the significance of both ER status and tumor grade. The initial multivariate analysis for DSS identified all of the factors that appeared in the initial DFS analysis, plus the variable age. Development of the interaction term erbB-2 × EGFR, which reflected the status of both erbB-2 and EGFR (−, −, −, +, +, and + +), markedly improved both DFS and DSS prediction (Table 3).

For lymph node-negative patients, only tumor size and grade were significant prognostic factors. The addition of erbB-2, EGFR, or Gt (as either continuous or dichotomous variables) failed to provide improvement in survival prediction (although the statistical power was decreased in this subset because of fewer events). Tumor grade was the only variable that provided a prediction of DSS.

For node-positive patients, the number of positive lymph nodes (log +1), tumor size, and ER status were each significant factors for both DFS and DSS. The addition of erbB-2 or EGFR as continuous or dichotomous variables improved survival prediction for both DFS and DSS. Gt (as a continuous or dichotomous variable) failed to improve survival when considered in isolation. The interaction term erbB-2 × EGFR was highly significant for predicting both DFS and DSS (P = 0.0003 and P < 0.0001, respectively) for these node-positive patients.

Based on proposed motility signaling interactions (the master switch hypothesis) between EGFR, erb-B2, and gelsolin, we examined the relationship between patient outcome and the composite variable erbB-2 × EGFR × Gt. This combined trivariate term improved survival prediction for both DFS and DSS, and its predictive value was beyond that observed using the erbB-2 × EGFR interaction term alone (Fig. 1; Table 4). erbB-2+, EGFR+, Gt+ breast cancer patients had a median DSS of 40 months, whereas erbB-2+ EGFR+ Gt− patients had a median DSS of 60 months. In contrast, in the erbB-2− EGFR− subset, gelsolin expression appeared to have the opposite effect, with Gt+ patients having a longer median DSS (<50% dying of disease) as compared with Gt− patients having a median DSS of 120 months. The apparent positive benefit of Gt expression in the erbB-2− EGFR− subset is likely not to be related to motility signaling (see the biological basis discussed below). The cellular localization of Gt also appeared somewhat different according to erbB-2 and EGFR status. In erbB-2− EGFR− tumors, gelsolin expression was typically diffusely cytoplasmic with occasional membranous accentuation (Fig. 2A). In erbB-2+ EGFR+ tumors, gelsolin was usually cytoplasmic with membranous accentuation. However, gelsolin expression was focally localized and accentuated in elongated cells identified at the periphery of cell clusters or in single invasive cells embedded in stroma. These microscopic features suggest that the Gt in the erbB-2+ and EGFR+ tumor cells is localized to the leading or invasive edge of cells (particularly at the margins of the tumors; Fig. 2B). This pattern of distribution has been reported by others using in vitro model systems.
DISCUSSION

We have examined 11 distinct clinical and pathological measures of prognostic significance in a cohort of 790 breast cancer patients with long-term follow-up. Our results confirm numerous studies that have associated high tumor grade, large tumor size, lymph node involvement, ER negativity, and erbB-2 and EGFR expression with poor outcome in breast cancer patients. We have also demonstrated gelsolin expression in 56% of tumors and a lack of expression in 44% of cases in this series. These observations extend in a major way the previous studies of gelsolin expression in breast cancers (23, 26). This study represents the first correlative study between gelsolin expression and patient survival.

The mechanisms by which erbB-2 expression and EGFR expression independently influence prognostic outcome in breast cancer patients are not known. The activation of growth factor receptors results in phenotypic alterations that include changes in cell shape, adhesion, and motility (42–44). Results from both in vitro motility and in vivo invasiveness assays have suggested that receptor tyrosine kinase-associated enhanced motility may account for the observed enhanced metastatic capacity (45, 46). Detailed evaluation of the steps involved in tumor cell metastases suggests that EGF, which binds to EGFR, is a major chemotactic cytokine in some model systems. Our data support these hypotheses and suggest that the poor prognosis of erbB-2/EGFR tumor-positive patients may be due to enhanced motility or invasiveness (47). The isolation of circulating breast cancer cells from the peripheral blood of patients has also demonstrated enriched populations of erbB-2+ cells, supporting this hypothesis (48). In addition, videomicroscopy analysis of tumor cells in vivo in mice has demonstrated an important role for EGF-stimulated motility and chemotaxis in the process of invasion and extravasation, critical elements of the metastatic cascade hypothesis (45, 46, 49). Coexpression of erbB-2 and EGFR receptors with heterodimer formation has also been shown to augment ligand signaling, causing a greater degree of shape change and motility than expression of either receptor alone (32, 48, 50–52).

Activation of erbB-2 and/or EGFR receptors leads to modulation of the complex and branching downstream signal transduction pathways. This pathway activation may contribute to both cell growth and enhanced cell motility, as depicted in Fig. 3 (50). Activation of ras and rac GTPases plays a critical role in mediating downstream motility events, such as membrane ruffling and cell protrusion (53). Phosphatidyl-inositol 3-kinase converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, affecting several proteins that interact with actin, including gelsolin (42, 54). PLCγ is also a required signaling element for motility downstream of EGFR activation (31, 51, 55, 56). Transcriptional events have also been recognized to be important for EGF-induced motility (53).

As a direct consequence of these signaling events, dynamic alterations in the cytoskeletal actin architecture occur, facilat-
...ing cell motility. Cellular actin exists as both monomers and polymerized actin filaments. The dynamic assembly of actin filaments is the driving force for many forms of cell motility. Filament formation is regulated by the availability of free “barbed” actin filament ends, to which a pool of actin monomers can rapidly add (57). The free ends are created either by the release of barbed end-capping proteins, such as gelsolin from existing filaments, or through de novo creation by the Arp2/3 complex (58, 59). Although the Arp2/3 complex is responsible for the intracellular motility of *Listeria* and other pathogens, its role in cellular motility under other circumstances is less certain. It is likely that both mechanisms contribute to cellular motility of human epithelia (22, 59).

Gelsolin is widely expressed in multiple cell types including the breast epithelium (Ref. 23; data not shown). Although gelsolin-null mice survive, they have multiple defects in cell morphology and motility including neutrophils, platelets, osteoclasts, and dermal fibroblasts (27, 28), as well as a delay in...

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**Table 3** Cox proportional hazards model of multivariate survival

<table>
<thead>
<tr>
<th></th>
<th>n (patients)</th>
<th>n (events)</th>
<th>$\chi^2$</th>
<th>$\Delta \chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Patients, DFS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LogLNpos + grade + size + ER</td>
<td>634</td>
<td>312</td>
<td>131.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LogLNpos + grade + size + ER + erbB-2</td>
<td>634</td>
<td>312</td>
<td>139.73</td>
<td>8.31</td>
<td>0.0039</td>
</tr>
<tr>
<td>LogLNpos + grade + size + ER + EGFR</td>
<td>634</td>
<td>312</td>
<td>137.15</td>
<td>5.73</td>
<td>0.0166</td>
</tr>
<tr>
<td>LogLNpos + grade + size + ER + gelsolin</td>
<td>634</td>
<td>312</td>
<td>140.42</td>
<td>9.01</td>
<td>0.0027</td>
</tr>
</tbody>
</table>

**LN-negative patients, DFS**

| Size + grade | 311 | 104 | 19.34 |               |       |
| Size + grade + erbB-2 | 311 | 104 | 19.83 | 0.50 |       |
| Size + grade + EGFR | 311 | 104 | 19.36 | 0.31 |       |
| Size + grade + gelsolin | 311 | 104 | 19.15 | 0.31 |       |

**LN-positive patients, DFS**

| LogLNpos + size + ER | 334 | 214 | 50.24 |               |       |
| LogLNpos + size + ER + erbB-2 | 334 | 214 | 63.79 | 13.56 | 0.0002 |
| LogLNpos + size + ER + EGFR | 334 | 214 | 61.14 | 10.90 | 0.001 |
| LogLNpos + size + ER + gelsolin | 334 | 214 | 63.07 | 12.84 | 0.0003 |
| LogLNpos + size + ER + erbB-2/EGFR | 334 | 214 | 50.24 | 0.00 |       |
| LogLNpos + size + ER + erbB-2/EGFR$^2$ | 334 | 214 | 68.83 | 18.60 | 0.0003 |
| LogLNpos + size + ER + erbB-2/EGFR$^3$ + gelsolin | 334 | 214 | 72.38 | 3.55 |       |
| LogLNpos + size + ER + erbB-2/EGFR$^3$ × gelsolin$^2$ | 334 | 214 | 84.49 | 12.11 | 0.0023 |

**LN-positive patients, DSS**

| LogLNpos + size + ER | 332 | 175 | 51.83 |               |       |
| LogLNpos + size + ER + erbB-2 | 332 | 175 | 72.48 | 20.65 | <0.0001 |
| LogLNpos + size + ER + EGFR | 332 | 175 | 67.22 | 16.24 | <0.0001 |
| LogLNpos + size + ER + gelsolin | 332 | 175 | 77.57 | 25.74 | <0.0001 |
| LogLNpos + size + ER + erbB-2 + EGFR | 332 | 175 | 77.01 | 4.52 | 0.0334 |
| LogLNpos + size + ER + erbB-2/EGFR$^2$ | 332 | 175 | 83.67 | 31.81 | <0.0001 |
| LogLNpos + size + ER + ierbB-2/iEGFR + gelsolin$^2$ | 332 | 175 | 80.72 | 3.15 |       |
| LogLNpos + size + ER + ierbB-2/iEGFR$^2$ × gelsolin$^2$ | 332 | 175 | 84.63 | 3.44 |       |

* $\Delta \chi^2$: change in the $\chi^2$ from the base model using the log-rank statistic; LogLNpos, transformed variable created by taking the log of the number of positive lymph nodes plus 1; ER status, coded variable 1 = <10 fmol/mg protein or <20% immunopositivity, 2 = ≥10 fmol/mg protein or ≥20% immunopositivity; erbB-2, continuous variable; EGFR, continuous variable; gelsolin, continuous variable; gelsolin$^2$, coded variable (5 = negative, 6 = positive); EGFR$^2$, coded variable (1 = negative, 2 = positive); erbB-2$^2$, coded variable (1 = negative, 2 = positive); MIB-1, continuous variable; ierbB-2/iEGFR$^2$, interaction term (1 = erbB-2 and EGFR negative, 2 = erbB-2 negative and EGFR positive, 3 = erbB-2 positive and EGFR negative, 4 = erbB-2 and EGFR both positive); erbB-2/EGFR$^2$ × gelsolin$^2$, interaction term created by multiply the interaction term ierbB-2/iEGFR and the coded term gelsolin; LN, lymph node.
breast development (29). Gelsolin-null dermal fibroblasts have markedly reduced motility in multiple assays, despite overexpression of rac GTPase as a compensatory response (28). Thus, gelsolin appears to be an obligate downstream effector of rac signaling for motility of dermal fibroblasts and other cell types. The critical role of gelsolin in receptor tyrosine kinase (e.g., EGF/EGFR)-induced motility has been demonstrated in several cell culture systems. Surface receptor signaling activates downstream signals from PLC\(_{6}\)/(31). The actin-binding activity of gelsolin is then regulated by both Ca\(^{2+}\) and phosphatidylinositol 4,5-bisphosphate as well as other phosphoinositides (22).

Fig. 1 Kaplan-Meier survival curves for lymph node-positive patients by Gt, erbB-2, and EGFR overexpression. DFS and DSS at a median of 16.3 years of follow-up.

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>DFS n</th>
<th>% Disease-free</th>
<th>DSS n</th>
<th>% Surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squares = erbB-2-, EGFR-, Gt+</td>
<td>107</td>
<td>51%</td>
<td>106</td>
<td>64%</td>
</tr>
<tr>
<td>Circles = erbB-2-, EGFR-, Gt-</td>
<td>84</td>
<td>31%</td>
<td>83</td>
<td>46%</td>
</tr>
<tr>
<td>Triangles = erbB-2+, EGFR+, Gt-</td>
<td>15</td>
<td>20%</td>
<td>15</td>
<td>27%</td>
</tr>
<tr>
<td>Diamonds = erbB-2+, EGFR+, Gt+</td>
<td>50</td>
<td>12%</td>
<td>50</td>
<td>18%</td>
</tr>
</tbody>
</table>

Fig. 2 Immunohistochemical staining for gelsolin in human breast cancers using anti-gelsolin antibody. Representative photomicrographs are shown. A, Gt+, erbB-2-, EGFR- invasive cancer. Gelsolin staining is cytoplasmic with membranous accentuation in some cells (small arrow). B, Gt+, erbB-2+, EGFR+ invasive breast cancer. Transendothelial invasion is demonstrated [a breast cancer cell is deformed as it invades (large arrow) an adjacent blood vessel (small arrow)]. Note the high expression and localization of gelsolin at the margin of the cell, where it appears to be entering the vessel.

The effect of Gt on survival in erbB-2/EGFR-expressing breast carcinoma patients fits well with this model of signal transduction pathways leading to cell motility. Gelsolin mediates erbB-2/EGFR receptor signaling and allows actin cytoskeletal changes to occur, resulting in enhanced invasion and motility. We have also recently reported that coexpression of erbB-2 and EGFR results in a high level of erbB-2 activation, as measured by binding of the phosphorylation-specific antibody PN2A in breast cancers (60). Once activated, this complex pathway enables the malignant cells to traverse architectural and structural boundaries (such as endothelial cells.
and tumor stroma). This pathway appears to require all three components to enable metastasis (activation of receptor tyrosine kinases, signaling intermediaries, and gelsolin expression). This is the first evaluation of this complex signaling pathway in human breast cancer patients to determine clinical relevance.

The concept that proteins involved in cell motility may have prognostic value is not new. Thymosin β6 was identified as a prognostic marker in early-stage prostate cancer (61). We have recently found that gelsolin expression was a significant prognostic factor in early-stage non-small cell lung cancer (30). The rho GTPase, which regulates focal adhesion and actin stress fiber formation in fibroblasts, has recently been identified as a critical protein for melanoma metastasis to the lung in a mouse tail vein injection model (62).

The lack of prognostic value for gelsolin in breast cancers not co-overexpressing erbB-2/EGFR is unexpected but fits with the observation that gelsolin is present in normal breast epithelial cells and is required for normal breast morphogenesis. In tumor cells without up-regulation of the erbB-2/EGFR receptors, gelsolin expression may serve as a marker, directly or indirectly, of cells with more limited growth and/or metastatic potential. The normal breast epithelium undergoes a cyclic process of proliferation, differentiation, and cell death. In breast tumor cells expressing gelsolin, gelsolin may serve as an effector of apoptosis, accelerating tumor cell death (63). Gelsolin can also affect lipid metabolism and lipid signaling pathways (64).

We hypothesize that early on during malignant transformation of breast epithelial cells, a reduction in gelsolin expression confers a positive survival benefit on the malignant cell (reduced apoptotic capacity and enhanced lipid growth signaling). At later periods of malignant progression, overexpression and activation of receptor kinase signaling pathways may render insignificant the negative effects of gelsolin expression. At the same time, cells that reexpress gelsolin acquire enhanced metastatic competence. Our data support this hypothesis in two ways. First, we observed an association between gelsolin expression and overexpression of EGFR and erbB-2. Second, we observed the negative prognostic significance of gelsolin expression in cases with erbB-2 and EGFR overexpression. Confirmation of these findings in a large prospective cohort of uniformly staged and treated breast cancer patients is desirable.

REFERENCES


2424 erbB-2, EGFR, and Gelsolin in Breast Cancer


Gelsolin as a Negative Prognostic Factor and Effector of Motility in erbB-2-positive Epidermal Growth Factor Receptor-positive Breast Cancers
