

# Gelsolin as a Negative Prognostic Factor and Effector of Motility in erbB-2-positive Epidermal Growth Factor Receptor-positive Breast Cancers<sup>1</sup>

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## ABSTRACT

**Purpose:** erbB-2 and epidermal growth factor receptor (EGFR) may mediate motility via signaling that enables changes in the actin cytoskeleton. A physical basis for this motility may depend on the coexpression of gelsolin, a  $M_r$  80,000 actin-binding protein.

**Experimental Design:** The expression of erbB-2, EGFR, and gelsolin was analyzed in 790 archival invasive breast cancers. These data were compared with histological, clinical, and outcome data (median follow-up, 16.3 years).

**Results:** Protein overexpression was observed in overlapping subsets of breast cancers (38% of cases were erbB-2+; 15% of cases were EGFR+; and 56% of cases were gelsolin+). Tumor gelsolin was associated with overexpression of erbB-2 and EGFR, as well as with an aggressive tumor phenotype. By univariate and multivariate analyses, tumor gelsolin alone was not a prognostic factor. Overexpression of all three factors significantly predicted poor clinical outcome by univariate and multivariate analyses. For example, in node-positive patients, coexpression of all three markers was associated with a 3-year disease-specific survival (as compared with erbB-2+, EGFR+, gelsolin– patients, who had a median survival of 6 years).

**Conclusions:** These data suggest that gelsolin coexpression may be an important additional prognostic factor in erbB-2+, EGFR+ breast cancer patients. We hypothesize that this is due to the role of gelsolin in mediating motility and invasion.

Received 2/2/01; revised 5/11/01; accepted 5/31/01.

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<sup>1</sup> Supported by NIH Grants CA44768 and HL54188.

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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,<sup>4</sup> are well known, although linkage between downstream effects and patient outcome are less well understood.

erbB-2 is a  $M_r$  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  $M_r$  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-

<sup>4</sup> The abbreviations used are: EGFR, epidermal growth factor receptor; 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.

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

Gelsolin is a  $M_r$  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  $\text{Ca}^{2+}$ ) and cuts them into smaller pieces. Gelsolin then caps these short actin filaments at the rapidly growing end. Gelsolin dissociates from the ends of actin filaments by lowered  $\text{Ca}^{2+}$  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 infiltrating 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. MIB-1-derived proliferation data were available on 777 of these breast cancers. MIB-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, 1:400; 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 (excluding 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*  $\geq 1\%$  immunostaining. New composite variables were also constructed to test interactions between erbB-2, EGFR, and gelsolin. To test the two-way interaction between erbB-2 and EGFR, the variable developed (erbB-2  $\times$  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 constructed (erbB-2  $\times$  EGFR  $\times$  gelsolin). erbB-2  $\times$  EGFR  $\times$  gelsolin had 6 degrees of freedom because there were no patients in the erbB-2–EGFR+ gelsolin– group. The prognostic results using the variable erbB-2  $\times$  EGFR  $\times$  gelsolin were then compared with the results using the variables erbB-2  $\times$  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 residuals against number of positive nodes suggested that this transformation 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  $\geq 20\%$  of tumor cells positive by immunohistochemistry (institutional cut point). p53 was entered into the statistical models as a binary variable  $\geq 1$ , where any staining was considered positive, 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, pathological, and immunohistochemical data. erbB-2 positivity was observed in 303 (38%) of breast cancers (range, 0.5–100% of cells were erbB-2+); of these, 222 showed  $\geq 10\%$  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 patients. 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-negative and 73 (21%) node-positive cancers. Gt positivity was observed in 172 (51%) node-negative tumors and 214 (63%) node-positive tumors.

Associations between erbB-2, EGFR, Gt, and other clinical and histological features of these patients are shown in Table 1. erbB-2 expression, EGFR expression, and Gt expression were correlated with each other. 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 dichotomous (negative or positive) variable or a three-way variable (negative, 1–3, and 4+) using the  $\chi^2$  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 immunostaining, 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 ( $P \leq 0.0002$  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

Factor	n <sup>a</sup>	erbB-2			EGFR			Tu Gt		
		r	95% CI	P	r	95% CI	P	r	95% CI	P
Age	792	-0.146	-0.21 to -0.08	<0.0001	-0.165	-0.23 to -0.10	<0.0001	-0.076	-0.15 to -0.01	0.0329
Grade	758	0.208	0.14-0.28	<0.0001	0.201	0.13-0.27	<0.0001	0.229	0.16-0.30	<0.0001
Size	791	0.073	0.00-0.14	0.0410	0.088	0.02-0.16	0.0137	0.061	-0.01-0.13	0.09
No. of positive LNs <sup>b</sup>	678	0.068	-0.01-0.14	0.08	0.070	-0.01-0.14	0.07	0.009	-0.07-0.08	0.81 <sup>b</sup>
ER status	787	-0.239	-0.30 to -0.17	<0.0001	-0.256	-0.32 to -0.19	<0.0001	-0.153	-0.22 to -0.08	<0.0001
p53	789	0.130	0.06-0.20	0.0003	0.182	0.11-0.25	<0.0001	0.105	0.04-0.17	0.0030
MIB-1	779	0.052	-0.02-0.12	0.14	0.112	0.04-0.18	0.0017	0.162	0.09-0.23	<0.0001
MI	790	0.061	-0.01-0.13	0.09	0.031	-0.04-0.10	0.38	0.129	0.06-0.20	0.0003
erbB-2	792	0.657	0.62-0.70	<0.0001	0.657	0.62-0.70	<0.0001	0.168	0.10-0.24	<0.0001
EGFR	792	0.168	0.10-0.24	<0.0001	0.138	0.07-0.21	<0.0001	0.138	0.07-0.21	<0.0001
Gelsolin	792	0.168	0.10-0.24	<0.0001	0.138	0.07-0.21	<0.0001	0.138	0.07-0.21	<0.0001

<sup>a</sup>n, number of patients; r, correlation coefficient; CI, confidence interval; ER status, negative (<10 fmol/mg protein or <20% immunopositivity) or positive (≥10 fmol/mg protein or ≥20% immunopositivity); MI, mitoses/10 high-powered fields; LN, lymph node.

<sup>b</sup>Using LN as a continuous variable. Significant associations between gelsolin expression and lymph node metastases were observed (metastases versus node negative, P = 0.0036; 0 versus 1-3 versus >3 lymph nodes with metastases, P = 0.0145) using the  $\chi^2$  method.

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  $erbB-2 \times EGFR$ , 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  $erbB-2 \times EGFR$  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, erbB-2, and gelsolin, we examined the relationship between patient outcome and the composite variable  $erbB-2 \times EGFR \times Gt$ . This combined trivariate term improved survival prediction for both DFS and DSS, and its predictive value was beyond that observed using the  $erbB-2 \times EGFR$  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.

Table 2 Factors associated with survival

Factor	n <sup>a</sup>	DFS			DSS		
		Odds ratio	95% CI	P	Odds ratio	95% CI	P
Age <50 yrs	185	1			1		
Age ≥50 yrs	587	0.85	0.68–1.06	NS	0.91	0.71–1.17	NS
Grade 1	68	1			1		
Grade 2	349	1.54	1.02–2.32	<0.0001	1.45	0.91–2.33	<0.0001
Grade 3	326	2.27	1.51–3.41		2.50	1.57–3.99	
Size ≤2 cm	341	1			1		
Size >2 cm	431	1.55	1.26–1.90	<0.0001	1.36	1.09–1.71	0.0075
LN negative	326	1			1		
LN positive	336	2.70	2.14–3.40	<0.0001	3.02	2.31–3.95	<0.0001
No. of positive LNs							
0	326	1			1		
1–3	183	1.97	1.50–2.59	<0.0001	2.07	1.50–2.84	<0.0001
≥4	153	3.98	3.06–5.19		4.68	3.47–6.32	
ER negative	337	1			1		
ER positive	430	0.70	0.58–0.86	0.0004	0.66	0.53–0.82	0.0002
p53 negative	543	1			1		
p53 positive	226	1.17	0.95–1.45	NS	1.26	0.99–1.59	NS
MIB-1 < median	376	1			1		
MIB-1 ≥ median	383	1.64	1.34–2.00	<0.0001	1.71	1.36–2.15	<0.0001
MI < median	363	1			1		
MI ≥ median	407	1.54	1.26–1.88	<0.0001	1.79	1.42–2.25	<0.0001
erbB-2 <40%	618	1			1		
erbB-2 ≥40%	154	1.35	1.07–1.71	0.0127	1.44	1.11–1.88	0.0057
EGFR negative	650	1			1		
EGFR positive	122	1.90	1.49–2.42	<0.0001	2.16	1.67–2.80	<0.0001
Gt negative	338	1			1		
Gt positive	434	1.08	0.89–1.32	NS	1.10	0.81–1.26	NS

<sup>a</sup> n, number of patients; ER status, negative (<10 fmol/mg protein or <20% immunopositivity) or positive (≥10 fmol/mg protein or ≥20% immunopositivity); MIB-1, <17.8 versus ≥17.8, MI, mitoses/10 high-powered fields <6 versus ≥6, erbB-2 cut point of 40% maximized the differences for survival; LN, lymph node; NS, nonsignificant.

## 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). Phosphatidylinositol 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, facilitat-

Table 3 Cox proportional hazards model of multivariate survival

	<i>n</i> (patients)	<i>n</i> (events)	$\chi^2$	$\Delta\chi^{2a}$	<i>P</i>
All Patients, DFS					
LogLNpos + grade + size + ER	634	312	131.41		
LogLNpos + grade + size + ER + erbB-2 <sup>1</sup>	634	312	139.73	8.31	0.0039
LogLNpos + grade + size + ER + EGFR <sup>1</sup>	634	312	137.15	5.73	0.0166
LogLNpos + grade + size + ER + EGFR <sup>2</sup>	634	312	140.42	9.01	0.0027
LogLNpos + grade + size + ER + gelsolin <sup>1</sup>	634	312	132.33	0.92	
All Patients, DSS					
LogLNpos + grade + size + ER + age	633	244	133.17		
LogLNpos + grade + size + ER + age + erbB-2 <sup>1</sup>	633	244	143.88	10.72	0.0011
LogLNpos + grade + size + ER + age + EGFR <sup>1</sup>	633	244	140.96	7.79	0.0053
LogLNpos + grade + size + ER + age + EGFR <sup>2</sup>	633	244	150.24	17.07	<0.0001
LogLNpos + grade + size + ER + age + gelsolin <sup>1</sup>	633	244	134.01	0.84	
LogLNpos + grade + size + ER + age + EGFR <sup>2</sup> + erbB-2 <sup>1</sup>	633	244	115.12	0.88	
LogLNpos + grade + size + ER + age + EGFR/erbB-2 <sup>3</sup>	633	244	153.33	20.16	0.0002
LN-negative patients, DFS					
Size + grade	311	104	19.34		
Size + grade + erbB-2 <sup>1</sup>	311	104	19.83	0.50	
Size + grade + EGFR <sup>1</sup>	311	104	19.36	0.02	
Size + grade + EGFR <sup>2</sup>	311	104	19.15	0.31	
Size + grade + gelsolin <sup>1</sup>	311	104	23.01	3.67	
LN-negative patients, DSS					
Grade	312	74	7.64		
Grade + erbB-2 <sup>1</sup>	312	74	7.71	0.07	
Grade + EGFR <sup>1</sup>	312	74	8.73	1.09	
Grade + EGFR <sup>2</sup>	312	74	7.64	0.00	
Grade + gelsolin <sup>1</sup>	312	74	7.86	0.22	
LN-positive patients, DFS					
LogLNpos + size + ER	334	214	50.24		
LogLNpos + size + ER + erbB-2 <sup>1</sup>	334	214	63.79	13.56	0.0002
LogLNpos + size + ER + EGFR <sup>1</sup>	334	214	61.14	10.90	0.001
LogLNpos + size + ER + EGFR <sup>2</sup>	334	214	63.07	12.84	0.0003
LogLNpos + size + ER + gelsolin <sup>1</sup>	334	214	50.24	0.00	
LogLNpos + size + ER + erbB-2/EGFR <sup>3</sup>	334	214	68.83	18.60	0.0003
LogLNpos + size + ER + erbB-2/EGFR <sup>3</sup> + gelsolin <sup>2</sup>	334	214	72.38	3.55	
LogLNpos + size + ER + erbB-2/EGFR <sup>3</sup> × gelsolin <sup>2</sup>	334	214	84.49	12.11	0.0023
LN-positive patients, DSS					
LogLNpos + size + ER	332	175	51.83		
LogLNpos + size + ER + erbB-2 <sup>1</sup>	332	175	72.48	20.65	<0.0001
LogLNpos + size + ER + EGFR <sup>1</sup>	332	175	68.07	16.24	<0.0001
LogLNpos + size + ER + EGFR <sup>2</sup>	332	175	77.57	25.74	<0.0001
LogLNpos + size + ER + gelsolin <sup>1</sup>	332	175	51.83	0.00	
LogLNpos + size + ER + erbB-2 <sup>1</sup> + EGFR <sup>1</sup>	332	175	77.01	4.52	0.0334
LogLNpos + size + ER + erbB-2/EGFR <sup>3</sup>	332	175	83.67	31.81	<0.0001
LogLNpos + size + ER + ierbB-2 <sup>2</sup> + iEGFR <sup>2</sup> + gelsolin <sup>2</sup>	332	175	80.72	3.15	
LogLNpos + size + ER + ierbB-2/iEGFR <sup>3</sup> + gelsolin <sup>2</sup>	332	175	84.463	3.44	
LogLNpos + size + ER + ierbB-2/iEGFR <sup>3</sup> × gelsolin <sup>2</sup>	332	175	97.07	12.91	0.0016

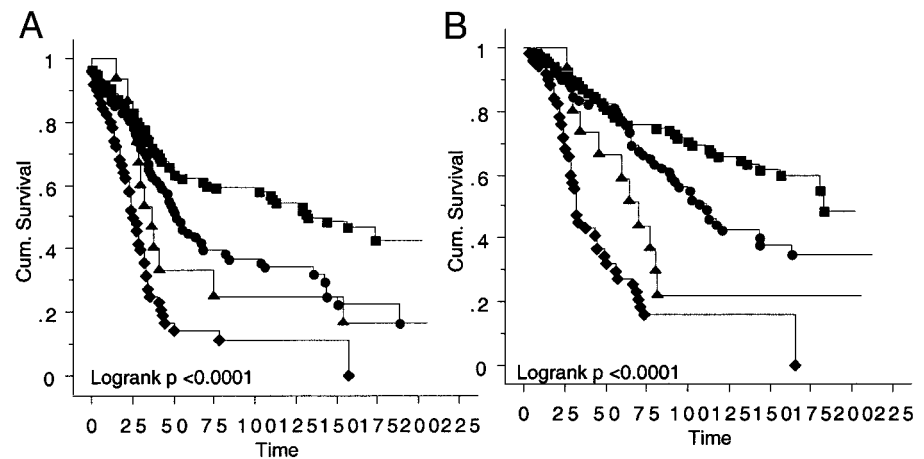
<sup>a</sup>  $\Delta\chi^2$ , change in the  $\chi^2$  from the base model using the log-rank statistic; LogLNpos, transformed variable created by taking the log of the number of positive lymph nodes plus 1; ER status, coded variable 1 = <10 fmol/mg protein or <20% immunopositivity, 2 = ≥10 fmol/mg protein or ≥20% immunopositivity; erbB-2<sup>1</sup>, continuous variable; EGFR<sup>1</sup>, continuous variable; gelsolin<sup>1</sup>, continuous variable; gelsolin<sup>2</sup>, coded variable (5 = negative, 6 = positive); EGFR<sup>2</sup>, coded variable (1 = negative, 2 = positive); erbB-2<sup>2</sup>, coded variable (1 = negative, 2 = positive); MIB-1, continuous variable; erbB-2/EGFR<sup>3</sup>, 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<sup>2</sup> × gelsolin<sup>2</sup>, interaction term created by multiply the interaction term ierbB-2/iEGFR and the coded term gelsolin; LN, lymph node.

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

**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.

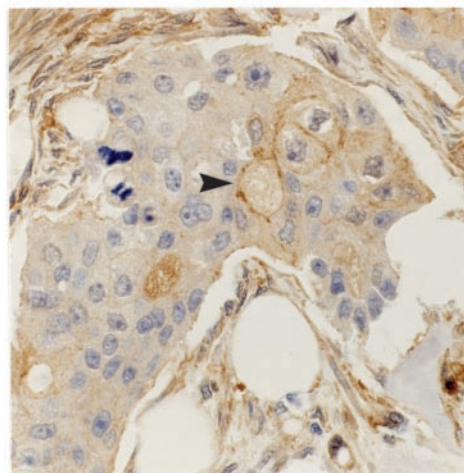


**Table 4** Patient groups and survival

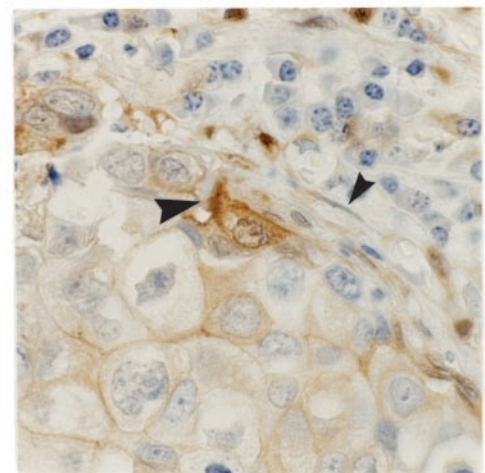
Patient groups	DFS n	% Disease-free	DSS n	% Surviving
Squares = erbB-2-, EGFR-, Gt+	107	51%	106	64%
Circles = erbB-2-, EGFR-, Gt-	84	31%	83	46%
Triangles = erbB-2+, EGFR+, Gt-	15	20%	15	27%
Diamonds = erbB-2+, EGFR+, Gt+	50	12%	50	18%

**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.

**A. Gelsolin +, erbB-2-, EGFR-**



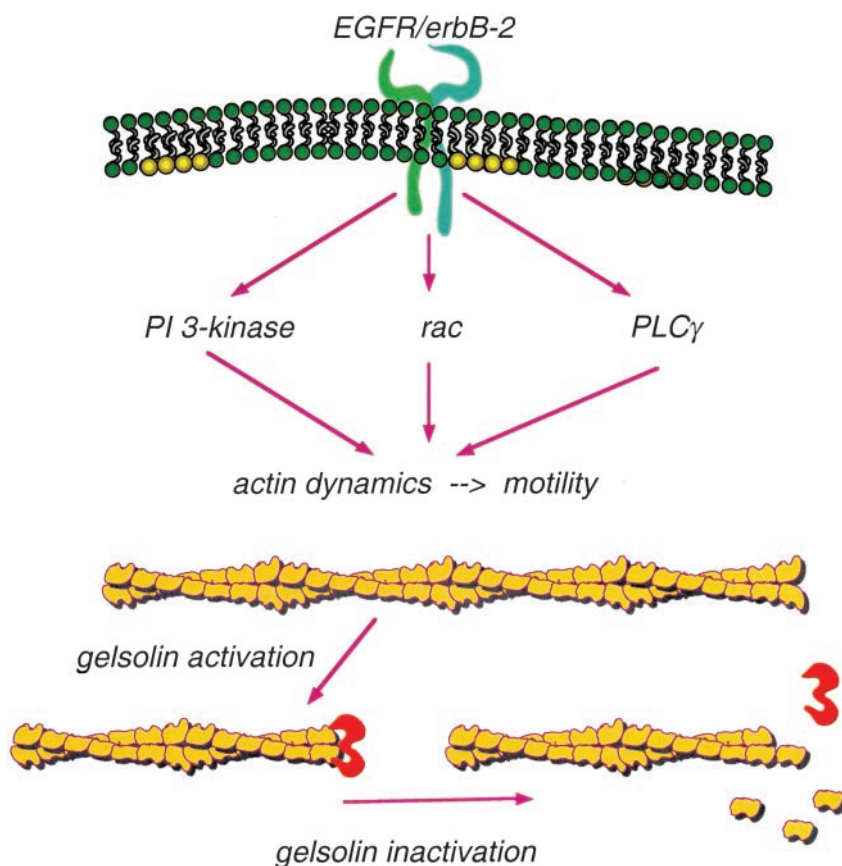
**B. Gelsolin+, erbB-2+, EGFR+**



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 $\gamma$  (31). The actin-binding activity of gelsolin is then regulated by both Ca<sup>2+</sup> and phosphatidylinositol 4,5-bisphosphate as well as other phosphoinositides (22).

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 anti-erbB-2 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





*Fig. 3* Schematic diagram of EGFR/erbB-2 activation leading to cellular motility. In this cellular model, the EGFR and erbB-2 receptors reside in the membrane and signal through phosphatidylinositol 3'-kinase (*PI 3-kinase*), the *rac* GTPase, and *PLC* $\gamma$  when stimulated. All three of these proteins cause dynamic changes in the actin cytoskeleton to effect cell movement. Gelsolin is an important mediator of this effect through its ability to reversibly sever actin filaments and provide sites for actin assembly.

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  $\beta$ 6 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.

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