# Bcar1/p130Cas Protein and Primary Breast Cancer: Prognosis and Response to Tamoxifen Treatment

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***Background:*** **The product of the Bcar1/p130Cas (breast cancer resistance/p130Crk-associated substrate) gene causes resistance to antiestrogen drugs in human breast cancer cells *in vitro.* To investigate its role in clinical breast cancer, we determined the levels of Bcar1/p130Cas protein in a large series of primary breast carcinomas.** ***Methods:*** **We measured Bcar1/p130Cas protein in cytosol extracts from 937 primary breast carcinomas by western blot analysis. The levels of Bcar1/p130Cas protein were tested for associations and trends against clinicopathologic and patient characteristics, the lengths of relapse-free survival and overall survival (n = 775), and the efficacy of first-line treatment with tamoxifen for recurrent or metastatic disease (n = 268).** ***Results:*** **Bcar1/p130Cas levels in primary tumors were associated with age/menopausal status and the levels of estrogen receptor and progesterone receptor. In univariate survival analysis, higher Bcar1/p130Cas levels were associated with poor relapse-free survival and overall survival (both two-sided *P* = .04; log-rank test for trend). In multivariate analysis, a high level of Bcar1/p130Cas was independently associated with poor relapse-free survival and overall survival. The response to tamoxifen therapy in patients with recurrent disease was reduced in patients with primary tumors that expressed high levels of Bcar1/p130Cas. In multivariate analysis for response, Bcar1/p130Cas was independent of classical predictive factors, such as estrogen receptor status, age/menopausal status, disease-free interval, and dominant site of relapse.** ***Conclusion:*** **Patients with primary breast tumors expressing a high level of Bcar1/p130Cas protein appear to experience more rapid disease recurrence and have a greater risk of (intrinsic) resistance to tamoxifen therapy. Thus, measurement of Bcar1/p130Cas may provide useful prognostic information for patients with primary or metastatic breast cancer. [J Natl Cancer Inst 2000;92:120–7]**

The breast epithelium is continuously exposed to the steroid hormone estrogen, which has been implicated in the proliferation of breast tumor cells and in the progression of breast cancer. Estrogens act by binding to the nuclear estrogen receptor (ER) present in the breast epithelial cells. The ER–ligand complex specifically activates target genes, resulting in a cascade of events leading to cell proliferation and differentiation *(1,2)*. The

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*See* "Notes" following "References."

recent cloning of a closely related ER, ERβ *(3,4)*, suggests the presence of alternative pathways of estrogen signaling. As yet, little is known about the role of ERβ in the development of human breast cancer *(5)*.

Endocrine therapies have been developed to block the effects of estrogen on cancer cells or to reduce serum estrogen levels. Tamoxifen is an antiestrogenic compound that is widely used as an adjuvant therapy for patients with ER-positive breast tumors *(6)*. The decision to use endocrine therapy for advanced breast cancer is based on a number of predictive factors, including ER or progesterone receptor (PgR) status, age, menopausal status, disease-free interval, and site of relapse. About 50% of the patients with advanced breast cancer receiving endocrine treatment have an objective response or stable disease (no change for >6 months). The rest of the patients have a short period of stable disease or immediate progressive disease *(7)*. The latter patients are considered to have intrinsic-resistant disease. However, almost all of the responding patients eventually experience disease recurrence (i.e., acquired resistance). Tamoxifen resistance may result from multiple mechanisms, and a number of causes have been suggested, including (epi)genetic alterations, resulting in ER mutants and splice variants *(8–10)*, and alteration of absorption, distribution, and metabolism of tamoxifen *(11,12)*. Elucidation of the mechanism(s) of tamoxifen resistance is, therefore, a comprehensive challenge in cancer biology.

To identify genes involved in hormone-independent growth, we used the technique of retroviral insertional mutagenesis and ZR-75-1 cells, a human breast cancer cell line. With this system, we have identified three loci responsible for breast cancer antiestrogen resistance (BCAR) *(13–15)*. The first genomic locus linked to the antiestrogen drug-resistant phenotype of the cells that we identified was the BCAR1 locus *(13)*. Characterization of the BCAR1 gene indicated that it is the human homologue of the rat-docking protein also known as p130 Crk-associated substrate (p130Cas) *(16)*. p130Cas, which contains an Src homology (SH) 3 domain, a proline-rich region, and a substrate domain containing multiple SH2-binding motifs, was identified as a major substrate for protein tyrosine phosphorylation in v-Src- and v-Crk-transformed cells *(17,18)* and has been implicated in a variety of biologic processes, including cell adhesion *(19,20)*, cell migration *(21–23)*, growth factor stimulation *(24)*, cytokine receptor engagement *(25)*, and bacterial infection *(26,27)*. Cell fusion-mediated transfer of the BCAR1 locus and transfection of BCAR 1/p130Cas complementary DNA into ZR-75-1 cells demonstrated that overexpression of Bcar1/p130Cas results in sustained cell proliferation in the presence of antiestrogens *(16)*. These observations prompted us to investigate the clinical relevance of Bcar1/p130Cas expression by measuring Bcar1/p130Cas protein in specimens of human breast carcinoma and testing these data against various characteristics of the patients and their disease for clinically relevant associations.

## Materials and Methods

### Patients and Tissues

Our study design was approved by the medical ethical committee of the University Hospital Rotterdam, The Netherlands.

**Nonmalignant breast tissues.** Breast tissue without specific abnormalities from 10 women who underwent reduction mammoplasty was used to study Bcar1/p130Cas levels in normal tissue. Three 50-μm cryosections from these tissues in TS buffer (i.e., 10 m*M* Tris–HCl [pH 7.5] and 1% sodium dodecyl sulfate [SDS]) were sonicated for 10 seconds and boiled for 10 minutes. Lysates were cleared by centrifugation at 12 000*g* for 10 minutes at room temperature, and total protein concentrations were determined with the BCA protein assay (Pierce Chemical Co., Rockford, IL). Bcar1/p130Cas levels were measured with the western blotting assay described below.

**Primary breast carcinoma.** Bcar1/p130Cas levels were determined in cytosol preparations (as described below) from 937 primary breast tumors collected from 1978 through 1988. Selection of samples was based on the availability of stored cytosol extracts (in liquid nitrogen), which remained after routine ER and PgR analyses. Of the 937 patients, 775 (modified mastectomy = 408 patients; breast-conserving treatment = 367 patients) were eligible for analysis of relapse-free survivors and overall survival according to the criteria described elsewhere *(28)*. Exclusion criteria were patient tissue that was taken from a biopsy specimen only (i.e., inoperable stage T4 tumors or tissue that was not obtained from the primary breast tumor) and patients with insufficient follow-up documentation. Patients who were referred to our institute more than 100 days after primary surgery and patients with distant metastasis at the time of primary surgery [M1 patients; staging according to the International Union Against Cancer TNM [tumor–node–metastasis] classification *(29)*] were excluded from analyses of relapse-free survival and overall survival, but they were not necessarily excluded from the analysis of response. In fact, we used 36 such patients in our analysis of the response to tamoxifen therapy in addition to those who were included according to the criteria described below.

Tumor size, lymph node status, and differentiation grade were gathered from the reports of regional pathologists. The differentiation grade is not based on a central pathologic review of all tumor samples and, thus, reflects daily practice. The median age of the 775 patients at surgery was 57 years (range, 27–90 years). Radiotherapy was given to 87% of the patients at one or more of the following sites: the breast/thoracic wall of 549 patients and/or the axilla of 296 patients and/or the lymph node areas other than the axilla of 328 patients. Adjuvant chemotherapy (mainly, cyclophosphamide, methotrexate, and 5-fluorouracil [CMF]) was given to 144 patients, and adjuvant hormonal therapy, either alone (38 patients) or in combination with CMF (14 patients), was given to 52 patients. All patients were routinely examined every 3–6 months during the first 5 years of follow-up and once a year thereafter. Of the 775 patients included in the study for analysis of relapse-free survival and overall survival, 442 (57%) showed evidence of relapse during follow-up, and 372 (48%) died. At the end of the study, 403 patients were alive, with a median follow-up of 105 months (range, 18–175 months). The following characteristics at surgery are shown in Table 1: tumor size, lymph node status, differentiation grade of the tumor, age, menopausal status, ER status, and PgR status.

Of the 442 patients who relapsed, 232 subsequently received tamoxifen therapy as a first-line treatment and were included in the analysis of response to first-line tamoxifen therapy for recurrent disease. The remaining patients were treated by surgery, radiotherapy, chemotherapy, or other forms of endocrine therapy or died without further treatment. The following inclusion criteria were used: patients with advanced disease who were treated with first-line tamoxifen therapy (40 mg/day) and were not exposed to hormonal treatment at an earlier stage (hormone naive). This subset was expanded to include the 36 patients excluded from analyses of relapse-free survival and overall survival. The median age of the patients with advanced disease at the start of tamoxifen treatment was 63 years (range, 28–91 years). Of these patients, 18% (n = 48) were premenopausal and 82% (n = 220) were postmenopausal. The dominant site of disease was the viscera in 107 patients, the bone in 118, and the soft tissue in 43. After primary surgery, 50 (19%) patients received systemic adjuvant chemotherapy (CMF, 41 patients; 5-fluorouracil, doxorubicin [Adriamycin], and cyclophosphamide [FAC], nine patients). During the course of their metastatic disease, 60% of the patients were subsequently treated with one or more additional hormonal treatments (mostly progestins) after disease progression during the first-line tamoxifen treatment. At the time of analysis, 40 patients were still alive, with a median follow-up of 38 months (range, 5–88 months), and 228 patients had died, with a median survival time of 20 months (range, 11 days to 8 years) after start of tamoxifen therapy. During follow-up but after the start of first-line endocrine therapy, 252 (94%) of 268 patients had disease progression, with a median time to disease progression of 5 months (range, 11 days to 6 years). Patients were examined during endocrine therapy at the outpatient clinic on average once every 6 weeks and during long-term remission up to once every 12 weeks. Response to treatment, as assessed by standard criteria, was defined as a patient having an objective response (complete [i.e., the complete disappearance of all metastases] and partial response) or prolonged stable disease, with a time

**Table 1.** Relationship of Bcar1/p130Cas expression with patient and tumor characteristics at the time of surgery

| Characteristic | Total* | Bcar1/p130Cas expression, % | | | | Two-sided *P* |
|---|---|---|---|---|---|---|
| | | None | Low | Intermediate | High | |
| Patients, No. | 775 | 33 | 39 | 20 | 8 | |
| Age, y | | | | | | .006† |
| ⩽40 | 101 | 37 | 41 | 19 | 4 | |
| 40–55 | 257 | 33 | 44 | 16 | 7 | |
| 56–70 | 270 | 33 | 35 | 21 | 11 | |
| >70 | 147 | 27 | 39 | 24 | 10 | |
| Menopausal status | | | | | | .04‡ |
| Premenopausal | 293 | 34 | 43 | 17 | 6 | |
| Postmenopausal | 482 | 32 | 37 | 22 | 10 | |
| Tumor size§ | | | | | | .86‖ |
| T1 ⩽2 cm | 294 | 32 | 39 | 19 | 9 | |
| T2 >2–5 cm | 372 | 32 | 40 | 20 | 8 | |
| T3/4 >5 cm | 97 | 35 | 38 | 20 | 7 | |
| Lymph node status§ | | | | | | .81‖ |
| N0 | 310 | 33 | 39 | 19 | 9 | |
| N1–3 | 224 | 33 | 40 | 20 | 7 | |
| N>3 | 232 | 31 | 41 | 20 | 9 | |
| Differentiation grade¶ | | | | | | .01‡ |
| Good/moderate | 140 | 23 | 45 | 24 | 8 | |
| Poor | 440 | 37 | 38 | 16 | 8 | |
| Missing | 195 | 30 | 38 | 24 | 8 | |
| Estrogen receptor status | | | | | | <.001† |
| Negative | 165 | 52 | 30 | 15 | 3 | |
| Positive | 606 | 27 | 42 | 21 | 10 | |
| Progesterone receptor status | | | | | | <.001† |
| Negative | 224 | 45 | 33 | 17 | 5 | |
| Positive | 535 | 27 | 42 | 21 | 10 | |

Because of some missing values, numbers do not always add up to 775 patients.
†Wilcoxon-type test for trend.
‡Mann–Whitney *U* test.
§For tumor size and lymph node status, *see (29).*
‖Kruskal–Wallis test.
¶Based on histologic and cellular characteristics, as stated in the reports of the regional pathologists.

to treatment failure of more than 6 months *(30–32).* Patients with prolonged stable disease and patients with a partial response have similar survival probability *(33,34).* When there was any doubt, the worst category of response was chosen. Of the 268 patients who received tamoxifen therapy as first-line treatment for recurrent or metastatic disease, 136 (51%) patients responded (42 had an objective response and 94 had stable disease). Of the 132 (49%) patients who did not respond, 17 showed no change in their disease for 6 months or less, and 115 showed progressive disease after treatment began.

## Preparation of Cytosolic Extracts From Tumors

Cryopreserved (liquid nitrogen) breast tumor specimens were pulverized with a microdismembrator as recommended by the European Organization for Research and Treatment of Cancer (EORTC) for determining cytosolic levels of the ER and PgR proteins *(35).* The resulting tissue powder was suspended in EORTC receptor buffer (10 m*M* $K_2HPO_4$, 1.5 m*M* $K_2EDTA$, 3 m*M* sodium azide, 10 m*M* monothioglycerol, and 10% [vol/vol] glycerol [pH 7.5]). The homogenate was centrifuged at 100 000*g* for 30 minutes at 40 °C, and the supernatant fraction (cytosol extract) was obtained and stored in liquid nitrogen until assayed. Total protein levels in the extracts were determined by use of the Bradford dye-binding assay (Bio-Rad Protein Assay; Bio-Rad Laboratories, Inc., Hercules, CA), with human serum albumin (Kabi Vitrum Diagnostica, Stockholm, Sweden) as the standard. ER and PgR levels were determined by a ligand-binding assay or an enzyme immunoassay as described previously *(36).* The cutoff point used to classify tumors as ER or PgR positive was 10 fmol of receptor protein/mg of cytosol protein.

## Western Blot Assay for Bcar1/p130Cas

To determine Bcar1/p130Cas levels in tissue, 5 μg of cytosolic or total extract protein from tumor tissue, nonmalignant breast tissue, or a cell line standard was boiled in loading buffer (i.e., 10 m*M* Tris–HCl [pH 6.8], 1 m*M* EDTA, 10% glycerol, 2% SDS, 2.5% 2-mercaptoethanol, and 0.00025% bromophenol blue) and electrophoretically separated in SDS–6% polyacrylamide gel. Each gel also contained prestained SDS–polyacrylamide gel electrophoresis molecular weight markers (Bio-Rad Laboratories) and lysate from the Bcar1/p130Cas-overexpressing human cell line 4A12 *(16)* in two concentrations as a standard. Proteins were transferred to a poly(vinylidene difluoride) membrane (Hybond P; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) with a semidry transblotter (Owl Scientific Inc., Woburn, MA). After an overnight blocking step at 4 °C in TBST (i.e., 100 m*M* Tris–HCl [pH 7.4], 1.5 *M* NaCl, and 0.05% Tween 20) containing 0.6% bovine serum albumin, the blots were incubated with a 1 : 5000 dilution of anti-p130Cas monoclonal antibody generated against rat p130Cas (Transduction Laboratories, Lexington, KY) for 1 hour. This procedure was followed by incubation with a 1 : 10 000 dilution of a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) for 1 hour. Immunocomplexes were visualized by chemiluminescence (enhanced chemiluminescence western blotting detection reagents; Amersham Pharmacia Biotech) and exposure to blue-light-sensitive x-ray screen film (CEA, Strängnäs, Sweden). To determine Bcar1/p130Cas protein levels, several exposure times (45 seconds, 90 seconds, 3 minutes, and 6 minutes) were used for each blot, and a Bcar1/p130Cas standard (two concentrations) was used as reference. Bcar1/p130Cas protein levels were scored visually by three investigators (S. van der Flier, A. Brinkman, and L. C. J. Dorssers) who were not aware of the clinical data. The consensus score was divided into the four following groups: none = no Bcar1/p130Cas protein expression detected on both short and long exposures of the film (e.g., *see* Fig. 1, lanes T1 and T9); low = no signal on a short exposure and a weak signal on a long exposure (Fig. 1, lane T4); intermediate = a weak signal on a short exposure and a clear signal on a long exposure (Fig. 1, lanes T2, T3, and T8); and high = a clear signal on a short exposure (Fig. 1, lanes T5, T6, and T7).

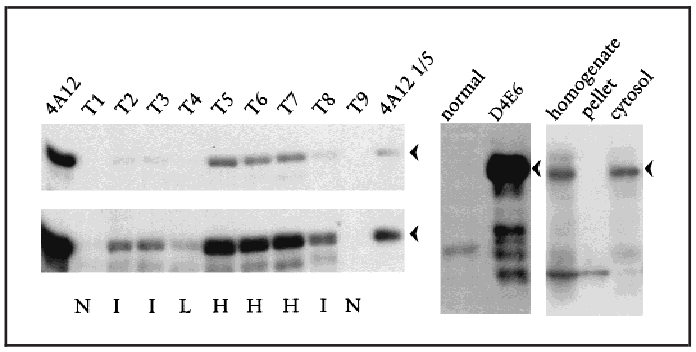


**Fig. 1.** Western blot analysis of protein extracts of human breast carcinomas and nonmalignant breast tissue. **Left panel:** Five micrograms of cytosol extracts from nine primary breast carcinomas (**lanes T1–T9**) was examined by western blot analysis with an anti-p130Cas monoclonal antibody (MAb) to measure Bcar1/p130Cas protein. As a standard, a total lysate from 4A12 cells, a Bcar1/p130Cas-overexpressing human cell line, was applied at two concentrations. Two exposure times of the relevant part of the blot are shown, with the position of the Bcar1/p130Cas band (approximately 116 kd) indicated by an **arrowhead.** Short (**top**) and long (**bottom**) exposures were used to classify Bcar1/p130Cas expression as none (**lanes N**), low (**lane L**), intermediate (**lanes I**), or high (**lanes H**). **Middle panel:** Five micrograms of a cell lysate from nonmalignant (normal) breast tissue was examined by western blot analysis with the p130Cas MAb. A very long exposure of the blot is shown. A cell lysate from D4E6 cells, a Bcar1/p130Cas-overexpressing cell line *(16),* was used as control. The **lower band** visible on the blot resulted from cross-reaction with the secondary antibody. **Right panel:** Equivalent amounts of the homogenate and cytosolic (cytosol) and pellet fractions from a primary breast tumor were examined by western blot analysis with MAb p130Cas.

### Statistical Analysis

The strength of the association between Bcar1/p130Cas levels in cytosolic extracts from tumors (treated as an ordinal variable) and other prognostic variables (patient or tumor characteristics treated as grouping variables) was tested with the Mann–Whitney *U* test or the Kruskal–Wallis test. To test for trends, a nonparametric test for trend across ordered groups as developed by Cuzick *(37)* was used. All *P* values are two-sided. Two-sided *P* values of less than .05 were considered to be statistically significant. Relapse-free survival and overall survival probabilities were calculated by the actuarial method of Kaplan and Meier *(38)*. A local disease recurrence (n = 442), distant spread (n = 313), or contralateral breast cancer (n = 37) was considered an event for relapse-free survival. For the analysis of relapse-free survival, patients who died without a reported disease recurrence were censored at their date of death. Both univariate and multivariate analyses were performed with the Cox proportional hazard model. For missing values, separate dummy variables were created. The assumption of proportional hazards was verified graphically. Relative hazard ratios were calculated and presented with 95% confidence intervals. For multivariate analysis, a basic model was introduced, including the classical prognostic factors (age, menopausal status, tumor size, number of positive lymph nodes, ER status, PgR status, differentiation grade, and systemic adjuvant therapy). Age and menopausal status combined were treated as one variable. The prognostic importance of Bcar1/p130Cas expression was tested by adding Bcar1/p130Cas to the basic model. The likelihood ratio test in the univariate Cox regression model was used to test for differences, and the log-rank test for trend was used to evaluate trends. The relation with response to therapy was examined with logistic regression analysis. For analysis of the time to disease progression after the start of tamoxifen treatment, the Cox proportional hazard model was used. All statistical analyses were performed with Stata Statistical Software (release 5.0; Stata Corporation, College Station, TX).

## RESULTS

### Detection of Bcar1/p130Cas Protein by Western Blot Analyses

The anti-p130Cas monoclonal antibody used recognizes the human Bcar1/p130Cas protein with an apparent molecular mass of 116 kd, as estimated by SDS–polyacrylamide gel electrophoresis *(16).* By analyzing subcellular fractions, we retained the bulk of Bcar1/p130Cas protein in routinely prepared cytosolic extracts (Fig. 1), thus enabling the screening of a large number of breast carcinomas for Bcar1/p130Cas expression. As shown by western blot analysis, levels of Bcar1/p130Cas protein differed in different tumors (Fig. 1). Bcar1/p130Cas protein expression was also evaluated in nonmalignant breast tissue from 10 women**.** In contrast to malignant tissue, Bcar1/p130Cas protein was not detected in any nonmalignant breast tissue analyzed (Fig. 1). To study the possible relationship with relapse-free survival and overall survival and response to first-line tamoxifen treatment, the expression levels of Bcar1/p130Cas from breast tumors were divided into four groups (none, low, intermediate, and high) based on the intensity of the western blot bands.

### Relationship of Bcar1/p130Cas Levels With Patient and Tumor Characteristics

We compared the levels of Bcar1/p130Cas protein in cytosolic extracts from 775 tumors with patient and tumor characteristics (Table 1). Thirty-three percent of the tumors had no Bcar1/p130Cas protein, 39% had low Bcar1/p130Cas expression, 20% had intermediate expression, and 8% had high Bcar1/p130Cas expression. Intermediate and high levels of Bcar1/p130Cas were more frequently found in tumors from older patients ($P = .006$). No statistically significant association of Bcar1/p130Cas levels with tumor size or lymph node status was observed. Reduced levels of Bcar1/p130Cas expression ($P = .01$) were statistically significantly associated with poorly differentiated tumors. Higher Bcar1/p130Cas levels were statistically significantly associated with ER- and PgR-positive tumors ($P<.001$). Expression levels of Bcar1/p130Cas were similar in tumors from patients who did not receive adjuvant treatment or who received adjuvant hormonal therapy or chemotherapy with or without hormonal therapy ($\chi^2 = 9.79$; $df = 6$; $P = .13$).

### Bcar1/p130Cas and Relapse-Free Survival and Overall Survival

We next studied the relationship between the level of Bcar1/p130Cas protein in primary tumors and the length of relapse-free survival. We found a statistically significant association of high Bcar1/p130Cas levels with poor relapse-free survival (log-rank test for trend; $\chi^2 = 4.38$; $df = 1$; $P = .04$) and poor overall survival (log-rank test for trend; $\chi^2 = 4.01$; $df = 1$; $P = .04$). Patients with low and intermediate levels of Bcar1/p130Cas protein in their primary tumor had similar periods of relapse-free survival (Fig. 2). On the basis of this observation, we decided to combine these levels for further analyses. Multivariate analysis was used to evaluate the possible independent prognostic importance of Bcar1/p130Cas expression in analyses of relapse-free survival and overall survival (Table 2). We introduced a basic model that included age, menopausal status, tumor size, number of positive lymph nodes, and the dichotomized biochemical parameters ER and PgR status. When we added the Bcar1/p130Cas level as an ordinal variable to the basic models for relapse-free survival and overall survival, we found that its expression level contributed to the prognostic information provided by the classical prognostic factors (Table 2). Thus, in patients with primary breast cancer, the level of Bcar1/p130Cas protein is an independent predictor of early recurrence and death.

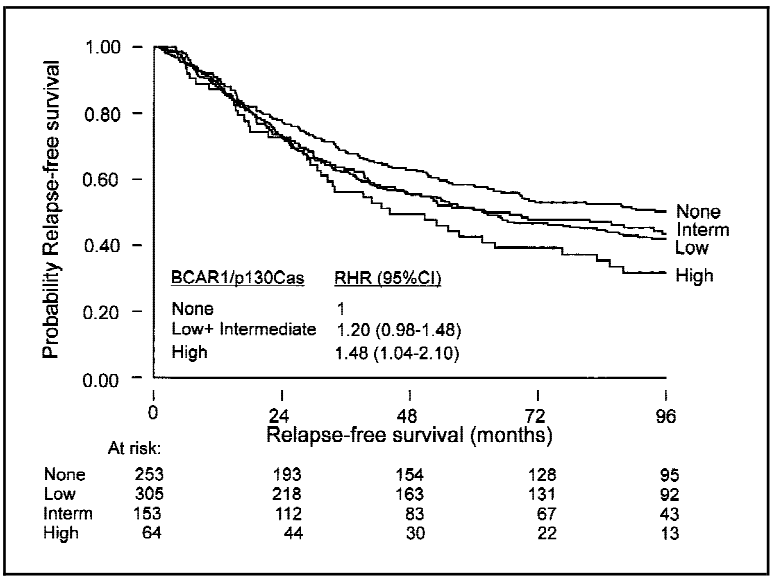


**Fig. 2.** Relapse-free survival of 775 breast carcinoma patients as a function of the expression level (none, low, intermediate [interm], or high) of Bcar1/p130Cas protein in the cytosolic extracts of primary breast carcinomas. The number of patients at risk are indicated. The survival probability (and their 95% confidence intervals [CIs]) at 48 and 96 months, respectively, are as follows: curve none = 0.63 (95% CI = 0.57–0.69) and 0.50 (95% CI = 0.44–0.56); curve low = 0.56 (95% CI = 0.50–0.61) and 0.42 (95% CI = 0.36–0.48); curve intermediate = 0.56 (95% CI = 0.47–0.63) and 0.44 (95% CI = 0.35–0.52); and curve high = 0.50 (95% CI = 0.37–0.61) and 0.32 (95% CI = 0.20–0.44). RHR = relative hazard rate; BCAR1/p130Cas = Bcar1/p130Cas.

### Bcar1/p130Cas and Response to Tamoxifen Treatment

Next, we investigated a possible relationship between the level of Bcar1/p130Cas protein in the tumor and the efficacy of response to tamoxifen as first-line systemic therapy for recurrent or metastatic disease. Of the 268 patients who were treated with tamoxifen, 136 (51%) responded, with a median time to disease progression of 16 months. The responses to tamoxifen were

**Table 2.** Multivariate Cox regression analysis*

| | Relapse-free survival | | Overall survival | |
|---|---|---|---|---|
| Factor | Two-sided *P* | RHR (95% CI) | Two-sided *P* | RHR (95% CI) |
| Basic model† | | | | |
| Age/menopausal status | .025 | | .21 | |
| Premenopausal‡ | | 0.76 (0.59–0.99) | | 0.91 (0.66–1.27) |
| Postmenopausal‡ | | 0.95 (0.82–1.10) | | 1.17 (1.01–1.36) |
| Postmenopausal versus premenopausal | | 1.09 (0.71–1.68) | | 1.01 (0.60–1.70) |
| Tumor size§ | .012 | | .002 | |
| T2 versus T1 | | 1.15 (0.92–1.43) | | 1.36 (1.05–1.75) |
| T3/4 versus T1 | | 1.68 (1.23–2.29) | | 1.95 (1.40–2.73) |
| Missing versus T1 | | 0.82 (0.33–2.01) | | 1.59 (0.73–3.47) |
| Lymph node status§ | <.001 | | <.001 | |
| N1–3 versus N0 | | 2.22 (1.66–2.97) | | 2.37 (1.72–3.26) |
| N>3 versus N0 | | 3.36 (2.54–4.44) | | 3.33 (2.46–4.52) |
| Missing versus N0 | | 2.04 (0.82–5.07) | | 1.83 (0.73–4.62) |
| ER status | .038 | | .036 | |
| Positive versus negative | | 1.18 (0.89–1.54) | | 0.99 (0.75–1.31) |
| Missing versus negative | | 5.06 (1.70–15.04) | | 5.77 (1.83–18.2) |
| PgR status | .76 | | .025 | |
| Positive versus negative | | 0.92 (0.72–1.18) | | 0.70 (0.54–0.90) |
| Missing versus negative | | 1.04 (0.52–2.09) | | 0.86 (0.43–1.73) |
| Systemic adjuvant therapy | <.001 | | .005 | |
| Hormonal therapy versus none | | 0.64 (0.42–0.99) | | 0.66 (0.42–1.03) |
| Chemotherapy ± hormonal therapy versus none | | 0.50 (0.37–0.69) | | 0.60 (0.42–0.85) |
| Differentiation grade¶ | .004 | | .26 | |
| Poor versus good/moderate | | 1.30 (1.00–1.70) | | 1.26 (0.94–1.69) |
| Missing versus good/moderate | | 0.90 (0.66–1.23) | | 1.12 (0.80–1.57) |
| Addition to basic model | | | | |
| Bcar1/p130Cas# | .03 | | .04 | |
| L + I versus none | | 1.20 (0.97–1.50) | | 1.24 (0.98–1.57) |
| High versus none | | 1.62 (1.13–2.32) | | 1.65 (1.11–2.46) |

*RHR = relative hazard rate; CI = confidence interval; ER = estrogen receptor; PgR = progesterone receptor; L = low; I = intermediate.

†Basic model included age/menopausal status (combined), tumor size, lymph node status, ER status, PgR status, systemic adjuvant therapy, and differentiation grade.

‡Age in decades tested separately for premenopausal and postmenopausal patients.

§For tumor size and lymph node status, *see (29)*

‖Chemotherapy with (n = 14) and without (n = 144) hormonal therapy.

¶*See* Table 1.

#Added to basic model. Final model included 775 patients.

similar in the subgroups with no (53%), low (50%), or intermediate (58%) levels of Bcar1/p130Cas protein in their tumors, whereas the response in patients with a high level of Bcar1/p130Cas in their tumors was lower (33%). However, the objective response was similar in all subgroups. The observed lower response rate in the high Bcar1/p130Cas subgroup is thus a reflection of a reduced number of patients with long-term stable disease. The tumors with high Bcar1/p130Cas levels were predominantly ER positive (29 [97%] of 30 tumors); fewer patients in the other subgroups had ER-positive tumors (approximately 85%). Again, the subgroups with low or intermediate Bcar1/p130Cas expression were combined. When analysis was performed on 230 ER-positive tumors only, there was a statistically significant association between a poor response to tamoxifen treatment and high expression of Bcar1/p130Cas (high = 34%; none = 61%; $P = .02$). In the multivariate analysis for response to treatment, Bcar1/p130Cas levels were corrected for menopausal status, relapse-free interval, and ER status (as a dichotomized variable). Independent predictors of poor response were premenopausal status, short disease-free interval, ER-negative status of the tumor, and high levels of Bcar1/p130Cas (Table 3). In a separate analysis, we included ER status as a continuous variable (log transformed) and found that a high level of Bcar1/p130Cas expression, compared with no expression, was still associated with a poor response to tamoxifen ($P = .01$). However, these results should be interpreted with some caution because we did not correct for multiple comparisons. An explanation for the observed differences between univariate and multivariate analyses could be the strong relation between an ER-positive status of the tumors and high Bcar1/p130Cas expression, which was corrected in the multivariate analysis.

The association between Bcar1/p130Cas expression and time to disease progression of the disease after the start of systemic treatment with tamoxifen, as analyzed by Cox regression analysis, was not statistically significant. Nevertheless, with increasing expression levels of Bcar1/p130Cas in the primary tumor, the median progression-free survival time of the patients was reduced substantially from 7 months (none, low, and intermediate expression) to 3 months (high expression).

## Discussion

An understanding of the molecular mechanisms of tamoxifen resistance should contribute to the determination of optimal therapies for individual patients. We have proposed that genetic alterations in the tumor cells allow these cells to escape antiestrogen therapy *(13)*. With our model system, we have identified the BCAR1 gene and shown that it can confer tamoxifen-resistant cell proliferation when overexpressed *in vitro (16)*. In this study, we investigated a possible association of the expression of Bcar1/p130Cas protein in primary human breast carcinoma with disease progression and response to tamoxifen therapy. Bcar1/p130Cas expression was found to vary considerably between the breast carcinomas, and relatively high Bcar1/p130Cas expression was detected in 8% of the breast primary tumors tested. In contrast, Bcar1/p130Cas protein was not detected in nonmalignant breast tissue samples by western blot analysis. Immunohistochemical analysis with the same antibody has confirmed that the Bcar1/p130Cas protein detected in cytosolic extracts of tumors by western blot analysis is localized in tumor cells and that the expression of Bcar1/p130Cas in normal breast tissue is observed in the limited numbers of ductal epi-

**Table 3.** Logistic regression analysis of response to tamoxifen therapy*

| | No. of patients | Univariate analysis | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | | %† | Two-sided *P* | Odds ratio | 95% CI | Two-sided *P* |
| All patients | 268 | 51 | | | | |
| Menopausal status | | | | | | |
| Premenopausal | 48 | 44 | | 1 | | |
| Postmenopausal | 220 | 53 | .02 | 2.54 | 1.26–5.09 | .01 |
| Dominant site of relapse | | | | | | |
| Soft tissue | 43 | 53 | | | | |
| Bone | 118 | 51 | .77 | — | | |
| Viscera | 107 | 50 | .66 | | | |
| Relapse-free survival, mo | | | | | | |
| <12 | 74 | 30 | | 1 | | |
| ⩾12 | 194 | 59 | <.001 | 3.29 | 1.79–6.06 | <.001 |
| ER status | | | | | | |
| Negative | 37 | 22 | | 1 | | |
| Positive | 230 | 56 | <.001 | 4.47 | 1.89–10.59 | <.001 |
| PgR status | | | | | | |
| Negative | 64 | 36 | | | | |
| Positive | 196 | 56 | .01 | — | | |
| Bcar1/p130Cas level‡ | | | | | | |
| None | 72 | 53 | | 1 | | |
| L + I versus none | 166 | 53 | .97 | 0.90 | 0.49–1.66 | .74 |
| High versus none | 30 | 33 | .08 | 0.38 | 0.14–0.98 | .04 |

*CI = confidence interval; ER = estrogen receptor; PgR = progesterone receptor; L = low; I = intermediate. There was one missing value for ER and eight for PgR.

†Percent overall response = percentage of patients with complete response, partial response, or stable disease for more than 6 months.

‡Distributions of Bcar1/p130Cas expression in tumors of the group of 36 patients included were not different from those of the remaining 232 patients selected from the disease-free survival analysis (Table 1) ($\chi^2 = 2.80$; $df = 3$; $P = .42$).

thelial cells and not in the abundant stroma (van der Flier S, Dorssers LCJ, van der Kwast TH: unpublished results).

Analysis of the expression of Bcar1/p130Cas protein in primary breast cancer has led to two distinct observations. First, relatively high expression of Bcar1/p130Cas in primary breast tumors is associated with a rapid recurrence of the disease. The risk of relapse was 62% greater for patients whose tumors had high levels of Bcar1/p130Cas than for patients whose tumors had undetectable levels of Bcar1/p130Cas protein. Multivariate analysis showed that a high level of Bcar1/p130Cas in the primary tumor was associated with an early relapse, independent of the classical prognostic parameters. Consequently, a high level of Bcar1/p130Cas protein in primary tumor extracts (compared with no detectable Bcar1/p130Cas in their primary tumor) is statistically significantly associated with a 65% reduced overall survival of patients. These results indicate that tumors with high-level expression behave more aggressively than tumors with low-level or undetectable expression. This association may be explained biologically by the involvement of Bcar1/p130Cas in cellular migration and invasion *(21–23)*. These properties suggest a higher risk of early dissemination of individual cells from the subgroup of tumors with high-level expression of Bcar1/p130Cas.

Second, high-level expression of Bcar1/p130Cas in the primary tumors (compared with tumors with no detectable Bcar1/p130Cas expression) is associated with a poorer response to first-line tamoxifen treatment. This observation is consistent with our findings with the *in vitro* model system; i.e., Bcar1/p130Cas overexpression resulted in antiestrogen resistance in cell lines *(13,16)*. Tamoxifen generally induces an objective response or stable disease in about half of the patients with ER-positive primary tumors and recurrent or metastatic disease *(39,40)*. The other patients who have ER-positive primary tumors experience immediate disease progression because of intrinsic resistance to tamoxifen. In our study, 67% of the patients with a relatively high level of Bcar1/p130Cas protein in their primary tumors did not respond to tamoxifen. This finding may explain some of the clinically observed intrinsic resistance to tamoxifen. Because these tumors are generally ER positive and the patients are, thus, eligible for tamoxifen therapy, Bcar1/p130Cas levels could be used by physicians to determine which patients may not respond to tamoxifen. Therapeutic decisions may benefit from more precise quantification of Bcar1/p130Cas protein levels in primary tumors. Because several lines of evidence show that development of resistance to antiestrogen agents is not always associated with the loss of ER expression in the tumor cells *(41–43)*, the association of the high levels of Bcar1/p130Cas in ER-positive primary tumors that have a poor response to tamoxifen suggests the possibility of an alternative pathway to bypass ER-mediated cell proliferation.

Despite many reports on Bcar1/p130Cas function in various cell systems, few clues are available about the pathway leading to the altered proliferation control in our cells *(44,45)*. Changes in adhesion and migration may affect cell proliferation, but the precise pathway in breast epithelial cells is yet unclear. Bcar1/p130Cas protein can be phosphorylated on tyrosine residues and can associate with many proteins that contain SH2 domains *(44,46,47)*. In addition, the SH3 domain of Bcar1/p130Cas may also associate with other proteins through proline-containing target sequences *(17,48,49)*. Bcar1/p130Cas serves as an adapter protein to contact proteins that participate in various cellular processes and is dependent on the availability, activation status, and affinity of its partners. These interactions are part of a cascade of signaling events leading to the regulation of gene expression and the regulation of the cell cycle. Because Bcar1/p130Cas appears to have a role in the progression of clinical breast cancer, elucidation of this critical pathway could contribute valuable information for the development of new treatment strategies for antiestrogen-resistant breast cancer.

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## NOTES

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