The Prognostic Value of BCAR1 in Patients with Primary Breast Cancer

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ABSTRACT

Purpose: BCAR1, the human homologue of the rat p130Cas protein, was identified in a functional screen for human breast cancer cell proliferation resistant to antiestrogen drugs. Here, we study the prognostic value of quantitative BCAR1 levels in a large series of breast cancer specimens.

Experimental Design: A specific ELISA was developed to measure BCAR1 protein levels in 2593 primary breast tumor cytosols. Tumor levels of BCAR1 were correlated with relapse-free survival (RFS) and overall survival (OS) and compared with collected data on urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1).

Results: In tumor cytosols, BCAR1 protein levels varied between 0.02 and 23 ng/mg protein. BCAR1 levels exhibited a positive correlation with steroid hormone receptor levels, age and menopausal status, and uPA and PAI-1 levels. The level of BCAR1 (continuous or categorized as low, intermediate, or high) was inversely related with RFS and OS time. Multivariate analysis showed that BCAR1 levels contributed independently to a base model containing the traditional prognostic factors for both RFS and OS (both \( P < 0.0001 \)).

When added together with uPA and PAI-1 in the multivariate model, BCAR1 contributed independently of PAI-1 and was favored over uPA. Interaction tests allowed for additional analyses of BCAR1 protein levels in clinically relevant subgroups stratified by nodal and menopausal status.

Conclusions: The quantitative BCAR1 protein level represents a prognostic factor for RFS and OS in primary breast cancer, independent of the traditional prognostic factors and the other novel marker PAI-1.

INTRODUCTION

Breast cancer will affect one in ten women in the Western world and 5-year survival may be expected for only \( \sim 60\% \) of the patients using current treatments. The heterogeneity of the disease additionally complicates the identification of patients with high risk for recurrence and disease progression and for sensitivity to systemic treatment. Many research efforts are focused on the identification of suitable markers for therapy selection for different risk groups of patients.

In a functional screen for genes involved in breast cancer resistance to antiestrogenic drugs, we previously reported the identification of the BCAR1 gene, which induces estrogen independence in estrogen-dependent human breast cancer cells (1). BCAR1 is the human homologue of the rat p130Cas gene, a prominent tyrosine phosphorylation target in transformed rat cells (2). Additional studies have shown that BCAR1/p130Cas is a cytoplasmic adaptor protein, capable of associating with many different proteins and contributing to various cellular processes, including adhesion, migration, cytoskeletal reorganization, host-pathogen interaction, survival, proliferation, transformation, and possibly metastasis (for review, refs. 3, 4). On the basis of semiquantitative Western blot detection of BCAR1 (using an antibody directed against rat p130Cas) in 775 breast tumor specimens, we previously showed that high levels of BCAR1 were significantly associated with early disease recurrence and with poor response to tamoxifen treatment of recurrent disease (5, 6). Because of the limitations of the Western blot assay, an extended analysis of the role of BCAR1 in breast cancer was hampered. The development of a quantitative BCAR1-ELISA (7) now offers the opportunity to address this question. Here, we report the prognostic value of BCAR1 protein levels in a large series of 2593 patients with primary breast cancer.

MATERIALS AND METHODS

Patients and Tissue Samples. BCAR1 levels were determined in cytosol preparations (as described below) from 2593 primary invasive breast tumors collected between 1978 and June 1995. Our study design was approved by the medical ethical committee of the Erasmus MC (Rotterdam, the Netherlands). Selection of samples for analysis of relapse-

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free survival (RFS) and overall survival (OS) according to the following criteria: at least 5 years of follow-up; no metastatic disease at diagnosis; no previous diagnosis of carcinoma, with the exception of basal skin carcinoma or cervical cancer stage 1; no evidence of disease within 1 month of surgery; and was additionally based on the availability of stored cytosol extracts (in liquid nitrogen) which remained after routine estrogen (ER) and progesterone receptor (PgR) analyses. Inoperable T4 tumors were not included. Tissue specimens that were sampled after neoadjuvant treatment, or obtained from a biopsy, were excluded. Patients with distant metastasis at the time of primary surgery (M1 patients; staging according to the International Union against Cancer Tumor-Node-Metastasis classification; ref. 8) were excluded.

Median age of the patients at the time of surgery (modified mastectomy = 1466 patients, breast-conserving treatment = 1127 patients) was 56 years (range, 22 to 90 years). One thousand sixty-three of them were premenopausal, and 1530 were postmenopausal at the time of primary surgery. Radiotherapy was applied to 1831 patients (71%): on the breast/thoracic wall in 1602 patients and/or on the axilla in 660 patients, respectively, parasternal and/or supraclavicular lymph nodes in 748 patients. T1 tumors (≤2 cm) were present in 1058 patients (41%), T2 tumors (>2 to ≤5 cm) in 1257 patients (48%), T3 tumors (>5 cm) in 170 patients (7%), and operable T4 tumors in 108 patients (4%). Pathological examination was not performed centrally and thus reflects daily practice in the participating regional hospitals as described previously (9). Histologic differentiation grade was coded as poor in 1379 patients (53%), moderate in 451 patients (17%), good in 40 patients (2%), and unknown for 723 patients (28%). None of the 1311 node-negative patients (51%) received systemic adjuvant therapy. Of the 1282 node-positive patients, 650 had one to three nodes (25% of total), and 632 patients had more than three nodes (24%) involved. Adjuvant chemotherapy alone (mainly cyclophosphamide/methotrexate/5-fluorouracil) was given to 438 patients (mainly premenopausal patients), whereas 322 patients received adjuvant hormonal therapy (mainly postmenopausal patients), either alone (294 patients) or in combination with chemotherapy (28 patients).

All patients were examined routinely every 3 to 6 months during the first 5 years of follow-up and once a year thereafter. The median follow-up period of patients alive (n = 1571) was 96 months (range, 1 to 221 months). Of the 2593 patients included, 1257 (48%) showed evidence of disease (including 212 locoregional relapses) and counted as failures in the analysis of RFS. One hundred fifty-two patients died without evidence of disease and were censored at last follow-up in the analysis of RFS. Eight hundred seventy patients died after a previous relapse. Thus, a total of 1022 patients (39%) failed in the analysis of OS.

**Assays of ER, PgR, Total Protein, Urokinase-Type Plasminogen Activator (uPA), Plasminogen Activator Inhibitor 1 (PAI-1), and BCAR1 in Tumor Tissue Extracts.** Tumor tissues were stored in liquid nitrogen and pulverized in the frozen state with a microdisembrator as recommended by the European Organization for Research and Treatment of Cancer for processing of breast tumor tissue for cytosolic ER and PgR determinations (10). The resulting tissue powder was suspended in European Organization for Research and Treatment of Cancer receptor buffer [10 mmol/L K2HPO4 buffer containing 1.5 mmol/L dipotassium EDTA, 3 mmol/L sodium azide, 10 mmol/L monothioglycerol, and 10% v/v glycerol (pH 7.4)]. The suspension was centrifuged for 30 minutes at 100,000 × g to obtain the supernatant fraction (cytosol). ER and PgR levels were determined by ligand binding assay or with enzyme immunoassay as described previously (9). The cutoff point used to classify tumors as ER or PgR positive and negative was 10 fmol/mg protein. The cytosolic levels of uPA and PAI-1 were determined with ELISAs as described before (11, 12). Total cytosolic protein was quantified with the Coomassie brilliant blue method (Bio-Rad Laboratories, Hercules, CA) with human serum albumin as a standard.

For quantification of BCAR1 in our ELISA, cytosolic breast cancer samples containing ~1 mg/mL protein were diluted 11-fold in PBS containing 1% BSA and 0.1% Tween 20 and incubated overnight at 4°C with microplate immobilized (with duck antichicken) capture chicken anti-BCAR1 antibody. After washing and trapping with rabbit anti-BCAR1 antibody, immune complexes were detected using goat antirabbit biotin-streptavidin-β-galactosidase (7). Different dilutions of a recombinant BCAR1 protein (calibrator) and a pool sample of identically prepared breast cancer cytosols (HR-1) were included in each series and used for quantification of BCAR1 and adjustment for day-to-day variation of the assay [HR-1 within and between assay coefficient of variation of 3.5% (range, 1.7 to 5.5%) and 4.5%, respectively]. The functional sensitivity of the assay was 4 pg/mL (7).

**Statistics.** The strength of the associations of BCAR1 with continuous variables was tested with Spearman rank correlation. The strength of the association of BCAR1 (used as a continuous variable) with other variables (used as grouping variable) was tested with the nonparametric Wilcoxon rank-sum test or Kruskal-Wallis test. Survival probabilities were calculated by the actuarial method of Kaplan and Meier (13). Both univariate and multivariate (i.e., multivariable) analyses were performed using the Cox proportional hazards model. We first defined the base model, including age, menopausal status, tumor and nodal status, grade, and ER/PgR status, and then investigated the interactions. To the base model, we next added separately BCAR1, uPA, and PAI-1 and checked the interactions. Because all three variables added significantly to the base model we included them simultaneously. By a step-down procedure for these added variables, we determined the final model (base model plus BCAR1 and PAI-1). The likelihood ratio test in the Cox regression models was used to test for differences and for interactions. In our search for the best categorization of BCAR1, we have used isotonic regression analysis (12, 14). In the univariate analysis for RFS, the proportionality assumption was investigated using a test based on the Schoenfeld residuals (15). In a generalized linear regression of the scaled Schoenfeld residuals on a function of time, the null hypothesis of zero slope is tested. All computations were done with the STATA statistical package, release 8.2 (STATA Corp., College Station, TX). All P values are two-sided.
RESULTS

BCAR1 Levels in Relation to Patient and Tumor Characteristics and uPA/PAI-1 Levels. The distribution of the levels of BCAR1 in 2593 tumor cytosols of primary breast tumors determined with the BCAR1-ELISA is presented in Fig. 1. The levels ranged from 0.02 to 23.0 ng/mg protein (median, 3.58 ng/mg protein). There appeared to be an approximately log-normal distribution pattern. Table 1 shows the median and interquartile range levels of BCAR1 in subgroups of tumors and their relationship with patient and tumor characteristics. The tumor level of BCAR1 was slightly lower in poor grade tumors, somewhat higher in postmenopausal patients, and was weakly positively related with age ($r_c = 0.06$, $P=0.002$), ER ($r_c = 0.18$, $P<0.0001$), and PgR ($r_c = 0.12$, $P<0.0001$). Compared with ductal, lobular, and mucinous carcinomas, medullary tumors contained lower levels of BCAR1 [$\chi^2 = 8.9$, degrees of freedom (df) = 3, $P=0.03$]. There were no significant correlations with tumor size status or lymph node status. BCAR1 levels were positively correlated with those of uPA ($r_c = 0.49$, $n = 2587$, $P<0.0001$) and PAI-1 ($r_c = 0.23$, $n = 2587$, $P<0.0001$; Table 1).

RFS and OS: Univariate Analysis. RFS and OS analyses as a function of log-transformed continuous BCAR1 levels showed statistically significant relationships with a poor prognosis in univariate analysis ($P=0.005$, $P=0.009$, respectively). This justified the search for (a) cut point(s) to allow visualization with survival curves and analysis as a categorical variable in addition to analysis of BCAR1 as a continuous variable. Using the results of the isotonic regression analysis (log-rank test for trend, $P<0.0002$; Fig. 2A) and OS ($P=0.0007$, Fig. 2B). Taking into account all failures in the analysis of RFS, the proportional hazards assumption was not violated, neither when using BCAR1 as a log-transformed continuous variable nor as a categorized variable.

RFS and OS: Multivariate Analysis. The independent relationship of BCAR1 with RFS and OS was studied with Cox multivariate regression analysis. In the analysis of RFS, which

![Fig. 1 Distribution of total BCAR1 over 2593 primary human breast tumor cytosols. Arrow indicates position of the median value at 3.58 ng/mg protein.](image)

![Table 1 Relationships of BCAR1 levels with patient and tumor characteristics](table)
included all 2593 patients, independent associations with poor prognosis were found for tumor size, nodal status, young pre- and postmenopausal age, premenopausal status, and poor tumor grade (Table 2). Steroid hormone receptor positivity (ER and/or PgR positive versus both negative) was not significantly related with RFS, which could be explained by a violation of the proportional hazards assumption (univariate $P < 0.0001$), a phenomenon described previously (16). In the multivariate analysis for OS, taking into consideration the traditional prognostic factors and the observed significant interactions between menopausal status and both nodal status and BCAR1 levels (see next paragraph), two differences were observed compared with the analysis of RFS, i.e., young postmenopausal age and steroid hormone receptor positivity were significantly related with a prolonged survival (Table 2). The multivariate model, which included age and menopausal status, tumor size, nodal status, tumor grade, and ER/PgR status, was defined as the base model (Table 2).

After inclusion of BCAR1 as a categorized variable in the multivariate models (Table 3), the increase in $\chi^2$ ($\Delta\chi^2$) was 28.3 and 23.0 (df = 2, $P < 0.0001$ for both) in the analyses of RFS and OS, respectively. Analysis of BCAR1 as a continuous variable again showed a significant contribution to the base models for RFS and OS ($\Delta\chi^2 = 12.2$, df = 1, $P = 0.0005$, and 18.4, df = 1, $P = <0.0001$). Also the established strong prognostic factors uPA and PAI-1 (17, 18) significantly added to base models for RFS and OS ($\Delta\chi^2 = 36.2$ and $\Delta\chi^2 = 24.5$ for uPA, respectively, and $\Delta\chi^2 = 54.2$ (df = 2) and $\Delta\chi^2 = 36.0$ (df = 2) for PAI-1, respectively. After adding BCAR1, uPA, and PAI-1 to the base models for RFS and OS, the contribution of uPA was no longer statistically significant, whereas BCAR1 and PAI-1 both independently contributed to the base models that included the traditional prognostic factors (Table 3). The $\Delta\chi^2$ (df = 4) as a result of the simultaneous addition of BCAR1 and PAI-1 to the base model for RFS was 72.4, which indicates a significantly better fit than after the addition of either factor alone ($\Delta\chi^2 = 28.3$ and 54.2, respectively, each with df = 2). Similarly in the analysis of OS, after the addition of BCAR1, uPA, and PAI-1 together, the contribution of uPA was no longer statistically significant.

**Fig. 2** RFS (A) and OS (B) as a function of total BCAR1 status in 2593 primary breast cancer patients. Patients at risk are indicated. Cut points used, 1.75 and 4.04 ng/mg protein.
Table 2  Cox multivariate analysis: base model

<table>
<thead>
<tr>
<th>Factor</th>
<th>RFS</th>
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<th>OS</th>
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<tr>
<td></td>
<td>HR*</td>
<td>P†</td>
<td>HR*</td>
<td>P†</td>
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<tr>
<td>Age and menopausal status‡</td>
<td></td>
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<tr>
<td>Age premenopausal§</td>
<td>0.71 (0.63–0.80)</td>
<td>&lt;0.0001</td>
<td>0.73 (0.64–0.85)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Age postmenopausal§</td>
<td>0.90 (0.82–0.98)</td>
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<td>1.24 (1.13–1.36)</td>
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<tr>
<td>Post- versus premenopausal</td>
<td>1.21 (0.94–1.55)</td>
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<td>1.46 (1.14–1.87)</td>
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<td>Tumor size</td>
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<td>2 to 5 cm versus ≤2 cm</td>
<td>1.39 (1.23–1.58)</td>
<td>&lt;0.0001</td>
<td>1.49 (1.29–1.73)</td>
<td>&lt;0.0001</td>
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<td>&gt;5 cm versus ≤2 cm</td>
<td>1.75 (1.44–2.11)</td>
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<td>1.90 (1.55–2.33)</td>
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<td>Nodal status</td>
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<tr>
<td>N₀,₁ versus N₀</td>
<td>1.29 (1.04–1.59)</td>
<td>&lt;0.0001</td>
<td>1.85 (1.57–2.17)</td>
<td>&lt;0.0001</td>
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<tr>
<td>N₀,₂ versus N₀</td>
<td>2.27 (1.86–2.77)</td>
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<td>3.22 (2.76–3.75)</td>
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<tr>
<td>Grade</td>
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<tr>
<td>Poor versus well/moderate</td>
<td>1.38 (1.17–1.63)</td>
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<td>1.36 (1.13–1.63)</td>
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<tr>
<td>Unknown versus well/moderate</td>
<td>1.19 (0.99–1.43)</td>
<td></td>
<td>1.14 (0.93–1.40)</td>
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<tr>
<td>ER/PgR (positive versus negative)</td>
<td>0.92 (0.79–1.06)</td>
<td></td>
<td>0.24 (0.19–0.27)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Interaction nodal status ×</td>
<td>0.002</td>
<td></td>
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<tr>
<td>menopausal status</td>
<td></td>
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<tr>
<td>N₀,₁ × post versus pre and/or N₀</td>
<td>1.44 (1.08–1.92)</td>
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<tr>
<td>N₀,₂ × post versus pre and/or N₀</td>
<td>1.56 (1.20–2.02)</td>
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</tbody>
</table>

NOTE. Final base models included 2593 patients. For RFS, HRs and confidence intervals are corrected for interaction between nodal and menopausal status. For OS, no interactions were observed.
* Numbers in parentheses, 95% confidence interval.
† P values from likelihood ratio test.
‡ Age and menopausal status combined.
§ Age in decades for pre- and postmenopausal patients.
¶ Positive, ≥10 fmol/mg protein; negative, <10 fmol/mg protein; ER/PgR, one or both positive versus both negative.
Abbreviation: HR, hazard ratio.

Table 3  Cox multivariate analysis: corrected for base model

<table>
<thead>
<tr>
<th>Factor</th>
<th>RFS</th>
<th></th>
<th>OS</th>
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<tbody>
<tr>
<td></td>
<td>HR†</td>
<td>P‡</td>
<td>HR†</td>
<td>P‡</td>
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<tr>
<td>+ BCAR1§</td>
<td></td>
<td></td>
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<tr>
<td>Intermediate versus low¶</td>
<td>1.14 (0.90–1.44)</td>
<td>&lt;0.0001</td>
<td>1.37 (1.03–1.82)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>High versus low¶</td>
<td>1.60 (1.27–2.02)</td>
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<td>1.81 (1.36–2.40)</td>
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<tr>
<td>+ BCAR1 (continuous)¶</td>
<td>1.45 (1.18–1.78)</td>
<td>0.0005</td>
<td>1.68 (1.33–2.14)</td>
<td>&lt;0.0001</td>
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<tr>
<td>+ uPA§</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
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<tr>
<td>Intermediate versus low**</td>
<td>1.14 (0.96–1.35)</td>
<td></td>
<td>1.13 (0.94–1.37)</td>
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<tr>
<td>High versus low**</td>
<td>1.56 (1.31–1.85)</td>
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<td>1.51 (1.24–1.83)</td>
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<tr>
<td>+ PAI-§</td>
<td></td>
<td>&lt;0.0001</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Intermediate versus low**</td>
<td>1.42 (1.24–1.62)</td>
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<td>1.26 (1.09–1.46)</td>
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<tr>
<td>High versus low**</td>
<td>2.19 (1.77–2.71)</td>
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<td>2.11 (1.67–2.66)</td>
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<tr>
<td>+ BCAR1 + PAI-†‡</td>
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<tr>
<td>Intermediate versus low¶</td>
<td>1.08 (0.85–1.37)</td>
<td>0.0003</td>
<td></td>
<td>&lt;0.0001</td>
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<tr>
<td>High versus low¶</td>
<td>1.51 (1.19–1.91)</td>
<td></td>
<td>1.73 (1.30–2.30)</td>
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<tr>
<td>PAI-1§</td>
<td></td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Intermediate versus low**</td>
<td>1.35 (1.18–1.55)</td>
<td></td>
<td>1.21 (1.04–1.40)</td>
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</tr>
<tr>
<td>High versus low**</td>
<td>2.04 (1.64–2.53)</td>
<td></td>
<td>1.98 (1.56–2.49)</td>
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</tbody>
</table>

NOTE. Base model as defined in Table 2. For RFS, HRs and CI are corrected for interaction between nodal and menopausal status and menopausal status and BCAR1 levels, if applicable. For OS, correction for interaction between BCAR1 and menopausal status, if applicable. All multivariate models with either uPA or PAI-1 included at least 2587 patients.
* Factors added alone or together to the base model.
† Numbers in parentheses, 95% CI.
‡ P values from likelihood ratio test.
§ Added alone to the base model.
¶ Low, ≤1.75 (n = 477); intermediate, >1.75 and ≤4.04 (n = 1023); high, >4.04 ng/mg protein (n = 1093).
|| Log-transformed variable.
** See Table 1.
†† Added together to the base model = final model.
Abbreviations: HR, hazard ratio; CI, confidence interval.
significant and BCAR1 and PAI-1 together added a $\Delta \chi^2 = 43.3$ (df = 4) compared with either factor alone ($\Delta \chi^2 = 23.0$ and $\Delta \chi^2 = 36.0$, both df = 2, respectively). Thus, for both the analysis of RFS and OS, BCAR1 independently contributed to the prognostic value of the established strong prognostic factor PAI-1, where BCAR1 was favored over uPA.

**Effects of Adjuvant Treatment and Other Interactions.**

Addition of adjuvant (none, chemo, or hormone) treatment as an indicator variable to the base model for RFS only marginally changed the estimates for categorized BCAR1 when added alone. As a result of the presence of adjuvant therapy in the base model, there was no significant difference for both the BCAR1-high group [hazard ratio (95% confidence interval) = 1.51 (1.19–1.91) versus 1.53 (1.21–1.93)] and the BCAR1-intermediate group (hazard ratio = 1.08 versus 1.04). Similarly, in the analysis of OS, no significant effects on the coefficients of BCAR1-intermediate or BCAR1-high were observed after adding adjuvant treatment to the base model. In addition, in the analysis of RFS in all patients or in the subgroup of node-positive patients, there were no statistically significant interactions between adjuvant endocrine- or chemotherapy with BCAR1 (included either as a categorized or as a log-transformed continuous variable). Furthermore, in the analysis for RFS, there were no statistically significant interactions between BCAR1 and nodal status, ER/PgR status, uPA, or PAI-1. In contrast, statistically significant ($P < 0.01$) interactions of menopausal status with nodal status and menopausal status with BCAR1 were observed in the analysis for RFS.

**BCAR1 and RFS in Subgroups of Patients.**

Because there was an interaction between menopausal status and nodal status, we considered the patients stratified by menopausal status and nodal status as four clinically distinct subgroups. Kaplan-Meier univariate analysis for RFS showed that in premenopausal/node-negative patients (Fig. 3A) and in postmenopausal/node-positive patients (Fig. 3D), higher BCAR1 levels were associated with a poor prognosis (for both, $P < 0.01$) with a difference in 10-year RFS between the extreme curves of 17 and 14%, respectively. A similar, nonsignificant trend was observed for postmenopausal/node-negative patients (Fig. 3B) and premenopausal/node-positive patients (Fig. 3C). Taken together, in the node-negative patients, there was a difference of 26% at 10-year RFS between the group performing best (69% for BCAR1-low in postmenopausal patients; Fig. 3B) and worst (43% for BCAR1-high in premenopausal patients; Fig. 3A). Furthermore, a striking difference was observed for the patients with tumors containing intermediate levels of BCAR1 when comparing the premenopausal patients (Fig. 3, A and B) with the postmenopausal patients (Fig. 3, B and D). For the premenopausal subgroups, the prognosis of the patients with intermediate BCAR1 levels is similar to that with low BCAR1 levels, whereas for the postmenopausal patients it is the same as in the group with high BCAR1 levels. These results for RFS are
also visualized in Fig. 4, left panel, in which the univariate hazard ratio of the patients with low levels of BCAR1 is set at 1.0, which is presented as a vertical line. In the analysis of OS as well, premenopausal patients with intermediate tumor levels of BCAR1 behave similarly as those with low levels, whereas in postmenopausal patients, they have a similar prognosis as those with high levels of BCAR1 (Fig. 4, right panel). Additional exploratory analyses for RFS in the clinically relevant subgroups of node-negative and -positive patients and ER-positive and -negative patients showed that the prognostic value of BCAR1 did not differ substantially between the nodal subgroups but that it was more prominent in ER-positive patients compared with ER-negative patients (Fig. 4, left panel). In the analyses for OS, the difference between the ER subgroups was less apparent (Fig. 4, right panel).

DISCUSSION

Previous evaluations of the role of the BCAR1 protein in breast cancer progression were hampered by the incomplete specificity of the antibodies directed against p130Cas in the semiquantitative and laborious features of the Western blot assay (5). Our current BCAR1 ELISA resolves both problems. The closely related HEF1 protein (also termed NEDD9), which has a similar domain structure as BCAR1 (4), is very well discriminated by the BCAR1 ELISA. HEF1 does not contribute to the signal of BCAR1 in our assay (7). This ELISA has facilitated the rapid quantitative analysis of the BCAR1 levels in a large series of breast tumor cytosols. The results presented here extend the results of our previous Western blot data (5). Direct comparison of the BCAR1 levels measured with semiquantitative Western blotting with the corresponding ELISA data showed good concordance ($r_s = 0.56$, $n = 592$, $P < 0.0001$) in a random set of cytosol samples measured with both assays (7). Association of BCAR1 levels with patient age and menopausal status and tumor differentiation grade and ER/PgR levels as previously reported (5) could be confirmed. A strong inverse relation between BCAR1 levels and RFS and OS after 10 years of follow up is evident in both univariate and multivariate analyses. Although uPA and PAI-1 contributed significantly to the base model as expected (18), simultaneous addition of BCAR1 causes loss of prognostic power of uPA likely because of the strong positive correlation between uPA and BCAR1 levels. Our data thus show that BCAR1 represents a prognostic factor independent of both traditional and strong novel prognostic factors (uPA and PAI-1). Because the tumor content of the tissue specimen may slightly affect the actual level of BCAR1, for future diagnostic studies, it may be beneficial to take the tumor percentage into account.

Dissection of the role of BCAR1 in the analysis of RFS in clinically distinct subgroups shows a correlation between BCAR1 levels and nodal status, ER status, and menopausal status. In these subgroups, higher BCAR1 levels are associated with increased hazard ratios (Fig. 4). Remarkably, patients with breast tumors containing intermediate levels of BCAR1 appear to have a distinct prognosis depending on their menopausal status (Figs. 3 and 4). In premenopausal patients, a similar prognosis is observed for tumors with low or intermediate BCAR1 levels. In contrast, in postmenopausal patients, tumors with intermediate and high levels have a similar poor prognosis. Different treatment modalities used for pre- and postmenopausal patients and node-negative and node-positive patients cannot explain this phenomenon. Similar profiles for the Kaplan-Meier RFS curves for each of the categorized BCAR1 levels were obtained in adjuvant treated and untreated patient subgroups (data not shown). Furthermore, no significant association between BCAR1 levels and adjuvant treatment was observed in any analysis.

The different prognosis of pre- and postmenopausal patients with intermediate tumor levels of BCAR1 is not caused by the slightly different median levels of BCAR1 (Table 1) because

![Fig. 4](https://example.com/f4.png)

Fig. 4  RFS (left) and OS (right) as a function of BCAR1 status in subgroups of patients stratified by nodal status, ER status, and menopausal status. The hazard ratio (HR) for tumors with low BCAR1 levels was set at 1.0 (vertical line). The data presented are the point estimates of the univariate HR and its 95% confidence interval (CI) (horizontal line). The number of patients in each BCAR1-intermediate and BCAR1-high subgroup is indicated. Cut points used, 1.75 and 4.04 ng/mg protein.
the distribution of patients among the BCAR1 categories is grossly similar (Fig. 3). We have previously shown that estrogen-independent breast cancer cell proliferation in vitro may be controlled by the level of BCAR1 protein (1). In the parental ZR-75-1 cells, the basal level of BCAR1 is insufficient to drive cell proliferation in the absence of estrogen. Either insertion of a retrovirus in the proximity of the BCAR1 gene or transfection of the BCAR1 cDNA into ZR-75-1 cells allows for estrogen-independent cell proliferation. We may speculate that increased levels of BCAR1 also contribute to the growth potential of breast tumors. Because changes in the hormone regulation are very prominent during menopause and tumor growth is at least in part dependent on estrogen levels (19), the contribution of BCAR1 to cell proliferation control may change accordingly. Alternatively, the change in hormonal regulation during meno-
earrowause may affect the role of BCAR1 in tumor metastasis. BCAR1 has been shown to be important in adhesion, motility, and migration of various cell types (3, 4, 20–25) and thus could increase the likeliness or success rate of tumor metastasis. The clear absence of an association between BCAR1 levels in the primary tumor and nodal status (Table 1) suggests that high BCAR1 levels increase the chances for disease recurrence independent of the nodal dissemination pathway.

Apart from the patient and breast tumor characteristics (menopausal status, nodal status, tumor size, and histology) and a limited number of well-established markers (ER and PgR), few newly identified marker proteins are applied for clinical decision making (26, 27). Prospective studies and/or meta-
earlyses are required to achieve this position (28). HER2/neu protein level and (or gene amplification) has proven to be important for selection of patients for specific treatment (29). Recently, meta-analysis of proteins of the plasminogen activator system has shown that high levels of uPA and PAI-1 are predictive for disease recurrence in patients with early breast cancer (17, 18, 30). The prognostic value of most novel markers appears insufficient to justify its single use in diagnosis. However, as exemplified by the prognostic power of the expression levels of a set of mRNAs predicting disease outcome (e.g., ref. 31), quantitative assays for a set of relevant proteins may provide detailed insight in the prognosis and treatment response of individual patients. Because of its independent prognostic value for RFS, BCAR1 may contribute in such a panel of analyses. In addition to prognosis, the predictive value of increased levels of BCAR1 in the primary tumor for poor clinical benefit of recurrent disease on first-line tamoxifen treatment (5) has been confirmed with the ELISA (32). Furthermore, our quantitative BCAR1-ELISA may prove useful for the analysis of small biopsy specimen of breast cancer and may also be applied to establish a prognostic value for BCAR1 in other malignancies and diseases. In addition to its association with human breast cancer, BCAR1/p130Cas has been linked to canine and feline mammary cancer (33) and suggested to contribute to malignant melanoma (34) and subsets of leukemia (35, 36). Overexpression of BCAR1/p130Cas in prostate cancer cells in vitro counteracts the inhibition of cell motility by CD82/KAI1 (37), suggesting that BCAR1/p130Cas plays a role in the metastasis suppressor function of KAI1 in prostate cancer (38) and other poorly differentiated or metastatic cancers (39). Expression of KAI1 was also found to be down-regulated in infiltrating breast cancer (40) and to be inversely associated with survival of breast cancer patients (41). Whether BCAR1 and KAI1 are counter-
earlyctive in breast cancer metastasis remains to be determined.

The results of our quantitative BCAR1 analysis within this large series of breast cancer patients indicate that the prognosis in the various clinically defined subgroups of patients varies according to the tumor BCAR1 levels and that BCAR1 may represent, in combination with other factors, a useful prognostic marker for patients with breast cancer.

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