Development of an ELISA for Measurement of BCAR1 Protein in Human Breast Cancer Tissue

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Background: High concentrations of breast cancer anti-estrogen resistance 1 (BCAR1) protein measured by Western blotting in primary breast tumor cytosols are associated with early disease progression and failure of tamoxifen therapy. The aim of the present study was to develop an ELISA to measure BCAR1 quantitatively in extracts of human breast cancer tissue.

Methods: A recombinant fragment of BCAR1 (the human homolog of murine p130Cas) was produced in bacterial M15 cells, purified, and injected into chickens and rabbits. The generated antibodies were affinity-purified and used for the construction of an ELISA. After validation, the results obtained with the ELISA were compared with Western blot findings on primary breast tumors.

Results: The detection limit the BCAR1 ELISA was 0.0031 µg/L, and the within-run imprecision (CV) was <20% at concentrations down to 0.004 µg/L. The within-run imprecision (CV) was 1.0–7.2%, and the between-run CV was 3.6–5.4%. There was no cross-reactivity with family member HEF1. The assay exhibited parallelism of results between serial dilutions and a mean recovery (range) of 96 (79–118)%.

Conclusions: The ELISA measures BCAR1 in human breast cancer cytosols with high sensitivity and specificity. The assay can be used to confirm and to quantitatively extend previous semiquantitative Western blot data on the prognostic and predictive value of BCAR1 in human breast cancer; it can also be applied for other diseases.

Metastasized breast cancer is a major cause of death in women. Both endocrine treatment and chemotherapy can delay progression of the disease, but ultimately fail because of the development of resistance to the treatment. The antiestrogen tamoxifen has been widely used for the management of estrogen receptor-positive breast cancer and is generally well tolerated by patients. Approximately one-half of patients will respond to the treatment. However, response duration is limited because of progression of the disease to an estrogen-independent phenotype. The mechanistic basis for treatment failure is as yet unknown and has provoked many studies.

We previously reported the identification of the breast cancer anti-estrogen resistance 1 gene (BCAR1)4 whose overexpression confers resistance against antiestrogen drugs to an estrogen-dependent human breast cancer cell line (1). In addition, we have shown that BCAR1 protein is present in (malignant) breast epithelial cells (2), and high concentrations of BCAR1 protein in 775 primary breast tumors were found to be associated with early disease progression. In a set of 268 patients, an association between high concentrations of BCAR1 protein in the primary tumor and failure of tamoxifen therapy for recurrent disease was observed (3). These investigations relied on semiquantitative Western blot analysis using an antibody directed against p130Cas, the rat homolog of BCAR1. BCAR1/p130Cas is an adapter protein involved in many different cellular signaling processes (4, 5). BCAR1/p130Cas shows extensive homology to the HEF1

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4 Nonstandard abbreviations: BCAR1, breast cancer anti-estrogen resistance; ITT, in vitro transcription and translation; and PBS, phosphate-buffered saline.
Materials and Methods

Expression constructs and protein production. Two expression constructs were generated from the previously described (1) human BCAR1 cDNA (accession no. AJ242987). To generate the first construct, which was intended for use in antibody production, we isolated the region encoding amino acids 272–541 (Fig. 1; NcoI-HindIII fragment B) and inserted it in Asp718 and HindIII-digested His-tag vector pQE-32 (Qiagen). The NcoI and Asp718 restriction sites were partially filled in to provide a single base-compatible end. Sequence-verified recombinant plasmid was isolated (pQE-B) and introduced in M15 (pREP4) bacterial cells for protein production (Qiagen). The resulting protein product was designated His-BCAR1/B.

The second expression construct, intended for use in antibody purification, was generated after PCR amplification of the fragment encoding amino acid residues 135–608 by use of primers F1 and R5 and cloning in pCR2.1-TOPO (Invitrogen). The amplified inserted fragment BCAR1/FIR5 was verified by sequence analysis, ligated into the GST-tag pGEX-3X (EcoRI), and introduced in Escherichia coli XL-10 cells. The resulting recombinant plasmid, pGEX-FIR5, was used for fusion protein (721 amino acids) production according to the protocols provided by the manufacturer. The obtained protein product was designated GST-BCAR1/FIR5. The same fragment, BCAR1/FIR5, was also ligated into the pQE-32 (BamHI/SalI), and the obtained pQE-BCAR1/FIR5 vector was used for production of His-tagged protein in E. coli XL-10 cells. The resulting protein (505 amino acids) was designated His-BCAR1/FIR5 and was used as the calibrator in the BCAR1 ELISA.

Additional protein preparations relating to BCAR1 were used in Western blotting experiments and in assay specificity studies. Some of these protein preparations were extracts from ZR-75-1 breast cancer cells and derived transfected cell lines. In addition to the parental cell line preparation, which produced a moderate amount of BCAR1, the study included the transfected 4A12, which overproduced BCAR1 (1), and transfectants that overproduced FLAG-tagged (Stratagene Europe) BCAR1 (F-BCAR1), HEF1, and a FLAG-tagged hybrid molecule consisting of the first 63 amino acid residues of BCAR1 and residues 64–834 of HEF1 (F-Hybrid). Stable overexpressing ZR-75-1-derived cell lines were produced as described previously for BCAR1 (1). Various protein preparations related to BCAR1 were generated by in vitro transcription and translation (ITT) procedures with use of a 17-RNA polymerase ITT reagent set (TNT Quick coupled transcription translation system; Promega) and expression constructs in pCR2.1-TOPO. These preparations included FLAG-tagged BCAR1 (ITT F-BCAR1), HEF1 (ITT HEF1), FLAG-tagged hybrid molecule (ITT F-Hybrid), and finally, BCAR2/TreP-132 (∼130 kDa), which was used as the negative control protein preparation (ITT Control).

Protein purification. Protein production in 100-mL bacterial cultures was induced with 1 mmol/L isopropyl-β-D-galactoside for 4 h according to the recommendation of the supplier. Recombinant His-BCAR1/B and His-BCAR1/FIR5 proteins were purified after lysis of the cells derived from ∼3 L of cultures by use of buffered 8 mol/L urea (pH 8.0) and affinity chromatography on Ni-NTA Agarose (Qiagen) as recommended by the supplier. Column-bound protein was eluted in buffered 8 mol/L urea (pH 4.5; 18 mL).

Recombinant GST-BCAR1/FIR5 protein was extracted from a 1-L culture of bacterial cells by sonication, loaded on glutathione-Sepharose 4B (Microspin GST purification module), and eluted with glutathione elution buffer (3 mL) according to the recommended protocol (Amersham Biosciences Europe GmbH). Elution fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with a Colloidal Coomassie Staining Kit (Invitrogen). Purified protein was quantified on stained gels against standardized quantities of bovine serum albumin (Pierce, Perbio Science).

Further purification and concentration of proteins was done by solid-phase extraction procedure using SPE® WP C4 columns from J.T. Baker. The process was monitored by measuring the absorbance of the fractions at 280 nm. After equilibration with the start buffer (0.67 g/L trifluoroacetic acid in water), original protein preparations were applied to the columns after extensive washing with the start buffer. After washing, proteins were eluted from the column with 2.5 mL of elution buffer (0.67 g/L trifluoroacetic acid in acetonitrile–2-propanol–water, 1:3:3 by volume). We estimated the amounts of BCAR1 obtained by measuring the absorbance of eluted fractions at 280 nm. The amounts were ∼0.5 mg for His-BCAR1/B.
protein (immunogen), ~0.4 mg for GST-BCAR1/F1R5 protein (affinity material), and ~0.7 mg for His-BCAR1/F1R5 protein (ELISA calibrator). Before further experiments, protein preparations were lyophilized in a SpeedVac® evaporator and reconstituted in 3–6 mL of phosphate-buffered saline (PBS).

Production of anti-BCAR1 polyclonal antibodies. Purified His-BCAR1/B protein was used for immunizing chickens and rabbits. Aliquots containing 10 μg of protein in 500 μL of PBS were mixed with an equal volume of Freund’s complete adjuvant for the first injection and Freund’s incomplete adjuvant for boosters. All injections in chickens and booster injections in rabbits were administered subcutaneously, whereas the first injection in rabbits was in the popliteal gland. Injections were repeated 12 times for both chickens and rabbits at 2-week intervals. Polyclonal antibodies against BCAR1, isolated from chicken egg yolks and citrate plasma from rabbits, were purified by affinity chromatography on GST-BCAR1/F1R5-coated columns (AffiGel®15; Bio-Rad Laboratories) according to previously described procedures (8, 10). The purified antibodies, marked 328# for chicken anti-BCAR1 and 329# for rabbit anti-BCAR1, were diluted with glycerol (1:1) and stored at −20 °C.

Western blotting. Immunologic detection of proteins by Western blot was performed essentially as described previously (3). Commercially available primary rabbit antibodies against p130Cas (N17Cas; Santa Cruz Biotechnology), the goat antibodies against HEF1 (N17HEF; Santa Cruz Biotechnology) and against the GST-tag (Amersham) together with the mouse antibodies against the RGS-His-tag (Qiagen) and against the FLAG-tag (CMZ; Stratagene) were diluted at least 1000-fold for use. The polyclonal antibodies against BCAR1, raised in chickens (328#) and rabbits (329#), were used diluted 300- to 10 000-fold. All secondary antibodies (coupled to horseradish peroxidase) directed against mouse IgG (Amersham), rabbit IgG (Amersham), chicken IgY (Sigma Chemical Co.), or goat IgG (DakoCytomation) were diluted at least 5 000-fold for use.

ELISA procedure. BCAR1 concentrations were measured by ELISA with the same experimental setup as described previously by Grebenschikov et al. (8). The assay format incorporated four different antibodies: (a) duck anti-chicken IgY antibody; (b) chicken anti-BCAR1 antibody; (c), rabbit anti-BCAR1 antibody; and (d) goat anti-rabbit antibody labeled with biotin.

The procedure started with coating of the microtiter plates (96-well Nunc Maxisorb™ flat-bottomed plates) with 100 μL/well of duck anti-chicken antibody (3.2 mg/L in 50 mmol/L NaHCO₃/Na₂CO₃, pH 9.6) overnight at 4 °C. The plates were then washed four times with 300 μL/well of washing buffer (1 mL/L Tween 20 in PBS) by use of a 96PW plate washer (SLT Lab Instruments GmbH). The plates were blocked with 10 g/L bovine serum albumin (cat. no. A-7906; Sigma) in PBS (300 μL/well) for 2 h at 37 °C and washed four times with washing buffer. The next step was the incubation with chicken anti-BCAR1 antibody [100 μL/well; 0.24 mg/L in dilution buffer (10 g/L bovine serum albumin in washing buffer)] for 2 h at 37 °C. After four washings, the calibrator (His-BCAR1/F1R5), the unknown samples, and the reference samples (100 μL/well in dilution buffer) were added to the wells and incubated overnight at 4 °C, after which the plates were again washed four times. All further incubations were performed at ambient temperature. After incubation with rabbit anti-BCAR1 antibody (100 μL/well; 0.18 mg/L in dilution buffer), performed for 2 h, the plates were washed and subsequently incubated with 100 μL/well of goat anti-rabbit biotin conjugate (cat. no. B-9642B; Sigma; 2500-fold diluted in dilution buffer) for 2 h and again washed. Thereafter, the plates were incubated with the streptavidin/β-galactosidase conjugate (cat. no. 1112481; Boehringer Mannheim; 2500-fold dilution in dilution buffer; 100 μL/well) for 2 h. The plates were then washed four times and incubated with 100 μL/well of 4-methylumbelliferyl-β-D-galactopyranoside conjugate (cat. no. M-1633; Sigma; 0.075 mmol/L in substrate buffer (100 mmol/L KH₂PO₄/K₂HPO₄ and 1 mmol/L MgCl₂, pH 7.4)) for 1 h. The reaction was stopped by the addition of 100 μL/well of 50 mmol/L NaHCO₃/Na₂CO₃ (pH 10.5), and fluorescence was measured with a fluorometric plate reader (Fluoroskan; Lab Systems) using 355 nm excitation and 460 nm emission filters.

Preparation of cell and tissue extracts and reference preparations. Total extracts of cell lines were prepared as described previously (3). Cytosol extracts from ZR-75-1-derived cell lines and tumor tissues were prepared and processed as recommended by the European Organization of Research and Treatment of Cancer (12), and quantitative estrogen receptor values were determined as described previously (13). Cytosol protein was quantified by the Coomassie Brilliant Blue method (Bio-Rad Laboratories) with human serum albumin as a standard. Two cytosol reference preparations (denoted 311000 and HR-1) were generated by pooling several breast cancer cytosols, preparing aliquots, and storing them at −80 °C. The lyophilized reference preparation, denoted 210800, was derived from xenografts raised in nude mice after implantation of MDA-MB-231 human breast cancer cells.

Validation of ELISA. Purified His-BCAR1/F1R5 was used as a calibrator in the BCAR1 ELISA to generate the dose–response curve. The detection limit was estimated as the minimum analytic concentration evoking a response significantly different from that of the zero calibrator. We also constructed plots of CVs of duplicate measurements (of several different cytosol samples and in-vitro-produced protein prepara-
Antibodies used in this study.

The cDNA fragment (NcoI–HindIII) was inserted in the pQE-32 expression vector, giving a bacterial fusion protein of 289 amino acids (pQE-32 His-BCAR1/B). The preparation His-BCAR1/F1R5, containing 0.13 g/L protein, were measured under the same conditions.

An additional test of assay specificity was performed by analyzing 40 heparin-plasma samples from healthy individuals, diluted 15-fold.

**Results**

**Expression constructs and protein production**

To develop antibodies specific for BCAR1, we selected a central part of the BCAR1 cDNA that encoded residues 272–541 of the protein (Fig. 1), which exhibited extensive sequence divergence with the related protein HEF1 (5). The cDNA fragment (NcoI–HindIII) was inserted in the pQE-32 expression vector, giving a bacterial fusion protein of 289 amino acids (~30 kDa), including a 6×His tag. This His-BCAR1/B protein was extracted with 8 mol/L urea buffer, affinity-purified on a Ni-NTA column, further purified by solid-phase extraction, and used for immunization of rabbits and chickens.

**Western blotting specificity**

After affinity purification on immobilized GST-BCAR1/F1R5, both chicken and rabbit anti-BCAR1 antibodies were tested for specificity on Western blots. The sodium dodecyl sulfate–polyacrylamide gel was loaded with total extracts from ZR-75-1 breast cancer cells together with ZR-75-1 cell-derived stable transfectants that overproduced BCAR1 or HEF1, and with in-vitro-produced BCAR1 and HEF1 (ITT BCAR1 and ITT HEF1). The rabbit anti-BCAR1 antibody (329#) displayed high specificity toward the in-vitro-produced target, with only modest cross-reactivity with the slightly smaller ITT-HEF1 protein (Fig. 2A, right lanes). In addition to the complete BCAR1 protein, partially degraded BCAR1 proteins were also detected by the antibody. The chicken anti-BCAR1 antibody (328#) identified the ITT-BCAR1 protein but produced more background on Western blot and also produced relatively stronger cross-reactivity signals with ITT-HEF1. Compared with the parental ZR-75-1 cell line, which produced moderate amounts of BCAR1, overproduction of BCAR1 in the total lysate preparation of the ZR-75-1 transfectant was readily detected by both antibodies (Fig. 2A, left two lanes). Low BCAR1 signals were also detected in two cytosol extracts from breast tumor specimens. In the lysate of ZR-75-1 cells transfected with HEF1, the endogenous BCAR1 protein band was efficiently detected, but no additional band was detected at the position of HEF1. Control goat anti-HEF1 antibody (N17HEF) recognized HEF1 produced in vitro as well in the appropriate overproducing transfectant. No cross-reactivity was observed in total extracts from the parental ZR-75-1 cells and the derived BCAR1 transfectant.

To further investigate the cross-reactivity with HEF1, we tested various in-vitro-produced proteins with the panel of antibodies (Fig. 2B). In addition to FLAG-tagged BCAR1 (ITT F-BCAR1) and HEF1 (ITT HEF1), we used FLAG-tagged chimerical protein of BCAR1/HEF1 (ITT F-Hybrid) and BCAR2/TreP-132 protein as a negative control (ITT Control). The hybrid protein contained the N-terminal SH3 domain from BCAR1 and the remainder of HEF1 (Fig. 1). As shown in Fig. 2B, both the FLAG-tagged BCAR1 and the hybrid proteins were nearly equally detected with mouse anti-FLAG antibody and rabbit anti-p130Cas antibody (N17Cas) directed against the extreme N-terminal part of BCAR1. In contrast, rabbit anti-BCAR1 antibody (329#) predominantly detected target protein and had only weak reactivity with HEF1 and the F-Hybrid protein. Goat anti-HEF1 antibody directed against the NH2 terminus of HEF1 (N17HEF) reacted only with the HEF1 product (Fig. 2B) with no cross-reactivity with either BCAR1 or the hybrid protein. None of these antibodies reacted specifically with the negative control protein.

**Detection limit and precision of the ELISA**

The preparation His-BCAR1/F1R5 was used as a calibrator to generate the dose–response curve of the BCAR1 ELISA. A typical calibration curve is shown in Fig. 3A together with the CVs for duplicate analyses. At all non-zero calibration points, CVs were ≤5.5%. The detection limit of the assay was 0.0031 µg/L (CV = 7.0%). The within-run imprecision (CV) was <20% at concentrations down to 0.004 µg/L (Fig. 3B).

To monitor long-term performance of the assay, we measured BCAR1 protein concentrations in three refer-
ence preparations included in all assays performed during the study. The concentration in reference preparation 311000 was 17.3 μg/L, with within- and between-assay CVs of 3.3% (range, 2.0–4.8%) and 5.4%, respectively. The reference preparation HR-1 contained 4.24 μg/L BCAR1 protein with within- and between-assay CVs of 3.5% (range, 1.7–5.5%) and 4.5%, respectively. Preparation 210800 contained 0.44 μg/L BCAR1 protein with within- and between-assay CVs of 4.4% (range, 1.0–7.2%) and 3.6%, respectively.

ELISA SPECIFICITY
The results of the measurements of different dilutions of several cytosol preparations based on ZR-75-1 breast cancer cell-derived stable transfectants are presented in Fig. 4A. The lowest concentration of BCAR1 protein (~22 mg/kg protein) was in the cytosol of the parental cell line ZR-75-1 and in the cytosol preparation of the transfectant that overproduced FLAG-tagged hybrid molecule (F-Hybrid). The highest concentration (~160 mg/kg protein) was found in the preparation that overproduced BCAR1 (BCAR1), and the cytosol preparation that overproduced FLAG-tagged BCAR1 (F-BCAR1) contained an intermediate concentration of BCAR1 (~60 ng/mg protein).

Analogous experiments performed with different ITT products (Fig. 4B) showed that only in the preparation with FLAG-tagged BCAR1 production (ITT F-BCAR1) was a high concentration of the analyte found, whereas in both the ITT HEF1 and ITT F-Hybrid preparations the concentrations of BCAR1 were very close to that of the negative control protein preparation (ITT Control). Because preparations ITT F-BCAR1 and ITT F-Hybrid both contained comparable amounts of protein (see Fig. 2B, signals obtained with N17Cas and FLAG antibody), our ELISA achieved adequate differentiation between BCAR1 and HEF1 proteins. No detectable amount of BCAR1 protein was observed in any of 40 studied heparin-plasma samples from healthy human individuals (results not shown).

LINEARITY AND RECOVERY
Three cytosol samples used in the linearity study were measured undiluted and diluted two-, three-, four-, and
fivelfold. The BCAR1 protein concentrations obtained in the undiluted samples were 0.19, 0.37, and 0.94 μg/L. For each sample, the dilution curve was close to linear, confirming parallelism between the calibrator and cytosol samples (Fig. 5).

To five cytosol samples (endogenous BCAR1 concentrations of 0.12–0.67 μg/L) we added the calibrator at 0.1, 0.2, and 0.4 μg/L. The recoveries ranged from 79% to 118% with a mean recovery of 96%.

**Comparison of BCAR1 Western Blot and ELISA of Primary Breast Tumors**

Of the 937 samples previously analyzed by Western blotting (3), two-thirds were available for quantification with the BCAR1 ELISA. We categorized the Western blotting results into four subgroups based on BCAR1 production: none detected, low, intermediate, and high. As shown in Fig. 6, we observed a strong positive correlation between the ELISA data and the Western blotting data ($r_s = 0.56; P < 0.0001$).
Discussion

BCAR1/p130Cas belongs to a small family of adaptor proteins shown to participate in many cellular processes (5). At least part of the functions of BCAR1/p130Cas cannot be substituted for by the family members HEF1 and EFS1 because homozygous deletion of the p130Cas gene causes embryonic lethality as a result of cardiovascular deficiencies (14). The roles of BCAR1/p130Cas and its numerous interaction partners have been investigated for the regulation of actin cytoskeleton, cell migration, and regulation of growth, apoptosis, and transformation [for a review, see Ref. (5)]. In addition, BCAR1/p130Cas has also been implicated in pathogen internalization, which depends on actin cytoskeleton rearrangement (15–17). We have identified BCAR1/p130Cas in a functional screen for anti-estrogen-resistant proliferation of human breast cancer cells and provided evidence that high concentrations of BCAR1 protein in primary breast tumors are associated with poor prognosis (1, 3). Furthermore, BCAR1/p130Cas has been associated with malignant melanoma and some types of leukemia (5) and was recently shown to interfere with KAI1/CD82-mediated suppression of metastasis in prostate cancer cells in vitro (18). Further evaluation of the role of BCAR1 in various kinds of malignancies and other diseases requires a specific and quantitative detection methodology.

In the present study we developed an ELISA for measuring BCAR1 concentrations in human breast cancer cytosols and cell line extracts. Western blotting, immunohistochemistry, and cell fractionation experiments have shown that the BCAR1 protein is located in the cytoplasm and is predominantly recovered in the cytosol fraction (2, 3). The BCAR1 ELISA exhibited a high analytical and functional sensitivity, low imprecision, and satisfactory parallelism and recovery. More importantly, the assay effectively differentiates BCAR1 from its family member HEF1, which is known to cross-react with other p130Cas antibodies. On Western blots, our polyclonal chicken and rabbit antibodies directed against the central part of BCAR1 still exhibited low cross-reactivity with in-vitro-produced HEF1 protein (Fig. 2). Apparently, structurally similar immunogenic/antigenic peptide fragments are generated despite the low primary sequence identity between these proteins in this region. As in the ELISA, two antibodies with very limited affinity for HEF1 are used; the resulting assay is highly specific for BCAR1. This specific BCAR1 ELISA should facilitate the analysis of BCAR1 concentrations in other (clinical) specimens, including tissues containing high amounts of the HEF1 protein.

In conclusion, the BCAR1 ELISA allows for specific measurement of BCAR1 protein in cell and tumor extracts and does not cross-react with the family member HEF1. Quantitative analysis of BCAR1 protein in breast tumor cy-
tosols showed good correlation with previous semiquantitative Western blotting.

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