Clinical Biochemistry 46 (2013) 1739–1746

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Characterization of estrogen response element binding proteins as biomarkers of breast cancer behavior

Traci L. Kruer [a,b], Timothy D. Cummins [c], David W. Powell [c], James L. Wittliff [a,b,*]

[a] Hormone Receptor Laboratory, Department of Biochemistry & Molecular Biology, Brown Cancer Center, University of Louisville, Louisville, KY 40292, USA
[b] Institute for Molecular Diversity & Drug Design, University of Louisville, Louisville, KY 40292, USA
[c] Department of Medicine, University of Louisville School of Medicine, Louisville, KY 40292, USA

ARTICLE INFO

Article history:
Received 1 November 2012
Received in revised form 27 June 2013
Accepted 1 July 2013
Available online 16 July 2013

Keywords:
Estrogen response element
Breast cancer
Biomarker

ABSTRACT

Background: While investigating estrogen response element (ERE) binding properties of human estrogen receptor-α (hERα) in breast cancer cytosols, other ERE-binding proteins (ERE-BP) were observed.

Design and methods: Recognition properties of ERE-BP were evaluated by electrophoretic mobility shift assays (EMSA) with ERE sequences of the 5′-flanking region of the estrogen responsive gene vitellogenin A2 (VitA2). Cytosols were incubated 16 h, 4 °C with [32_P]ERE sequences and separated by EMSA. A method of estimating ERE-BP levels was developed by measuring band intensity from EMSA profiles, expressed in digital light units (DLU)/μg protein and normalized to total DLU. ERE-BP were purified by affinity chromatography and EMSA, and then identified by mass spectrometry.

Results: ERE-BP in cytosols did not supershift in the presence of anti-hERα or anti-hERβ antibodies recognizing different ER epitopes suggesting that they are not fragments of either receptor isoform. ERE-BP competed with hERα for binding to VitA2–ERE. Increased levels of ERE-BP DNA-binding activities measured in 310 cytosols prepared from breast cancer biopsies correlated with decreased patient survival. Strikingly, breast cancer patients with ER negative status and high ERE-BP expression exhibited the poorest disease-free and overall survival. After purification, ERE-BP were identified as Ku70 (XRCC6) and Ku80 (XRCC5) using mass spectrometry. ERE-BP were confirmed to be Ku70/80 by supershift assay.

Conclusion: Presence of these novel ERE-binding proteins in a breast carcinoma is a strong predictor of poor prognosis. Our results suggest that ERE-BP, identified as Ku70/Ku80, in cytosols prepared from breast carcinoma biopsies are useful biomarkers for assessing risk of breast cancer recurrence.

Introduction

Carcinoma of the breast is the most common cancer affecting women in the United States with over 200,000 new cases estimated in 2013 and more than 39,000 deaths predicted [1]. It is the second most common cause of cancer-related death in women, and the main cause of death in women ages 40 to 59, although the incidence has been decreasing by 1.8% per year from 1999 to 2007 [1,2]. Current risk estimates suggest that a woman who lives to the age of 90 has a 1 in 8 chance of developing breast cancer [3].

Sporadic breast carcinomas comprise the majority of all breast cancer cases. For this phenotype of breast cancer, the major risk factors appear to be related to estrogen exposure [3]. The majority of sporadic breast cancers occur in post-menopausal women and more than 60% of the lesions over-express the estrogen receptor protein (ER) [3,4]. Many studies have examined the mechanisms by which estrogens influence differentiation and proliferation of cells that may result in aberrant signaling through estrogen-dependent pathways, thought to be a major mechanism for hormone receptor positive breast cancers [4–7]. However, additional mechanisms play a role in sporadic cancers as a significant number of breast carcinomas are ER negative [8].

In the classical pathway of estrogen action, ER alters the expression of target genes by binding to regions upstream of general promoters known as estrogen response elements (EREs). This mechanism affects the expression of genes involved in cell growth, proliferation and differentiation [9]. When either 17β-estradiol or another estrogenic ligand binds to ER, it induces a conformational change in the receptor protein leading to dimerization and binding to an ERE sequence [10,11]. The consensus ERE is a 13 bp palindromic inverted repeat with the sequence 5′-GGTCAnnnTGACC-3′; however ERα also may bind to imperfect ERE sequences [12,13]. The first ERE sequence was discovered in the 5′-flanking regions of the estrogen controlled vitellogenin genes of *Xenopus laevis* [14]. After ERE binding, a protein complex is formed with either coactivator (e.g. SRC-1, TIF1, TIF2, TFIIB) or corepressor proteins (e.g. NCoR, SMRT, SHP, REA) which bind to the dimer, culminating in either recruitment of general transcription factors to promote transcription of the adjacent gene or histone deacetylases to negatively affect ER-mediated transcription [15,16].

* Corresponding author at: Department of Biochemistry and Molecular Biology, HSC-A, Rm. 513, 319 Abraham Flexner Way, Louisville, KY 40202, USA. Fax: +1 502 852 8008.
E-mail address: jim.wittliff@louisville.edu (J.L. Wittliff).

0009-9120/$ – see front matter
http://dx.doi.org/10.1016/j.clinbiochem.2013.07.005

While investigating DNA-binding properties of ERα in breast cancer cytosols, we discovered proteins that recognized ERE sequences that did not appear to be either ERα or ERβ [17]. Our preliminary investigations suggested that the presence of these proteins in a biopsy was correlated with decreased survival probabilities in breast cancer patients. The purpose of this investigation was to characterize the proteins observed in breast tumor extracts that bind to ERE sequences in order to determine their role in breast cancer behavior and clinical value for assessing risk of recurrence and overall survival. Better understanding of the relationship between ERE-BP DNA-binding activities in tissue biopsies and breast cancer behavior will help define these proteins as potential prognostic biomarkers or therapeutic targets.

Material and methods

Preparation of human recombinant estrogen receptor (rhER) protein

The yeast strain BJ3505 was transformed with an hERα expression plasmid YEpE12. hERα is expressed as an ubiquitin fusion under the control of the CUP1 promoter [18,19]. Yeast cells were grown in complete minimal dropout medium without tryptophan and monitored spectrophotometrically at a wavelength of 595 nm. When the OD595 reached 0.6, rhERα expression was induced by the addition of CuSO4 to a final concentration of 100 μM. After harvesting and washing with water, yeast pellets were extracted in 40 mM Tris buffer, pH 7.4 with 1.5 mM EDTA, 10% glycerol, 10 mM Na_2MoO_4, 10 μM monothioglycerol and 1 mM PMSF. Yeast cells were disintegrated with glass beads in a Vortex Genie® in 5 intervals of 30 s each, with 30 s intervals cooling on ice. Debris was removed from the preparation by centrifugation at 100,000 ×*g* for 30 min at 4 °C. The supernatant containing rhERα was recovered for immediate assay or stored at −80 °C for future use as a reference representing a well characterized ERE-binding protein. rhERβ was purchased from Panvera (Invitrogen).

Cytosol preparation

Cytosols were prepared from de-identified human breast cancer tissue specimens in the same buffer described above and homogenized with a Polytron PT-10-35. Homogenate was separated into pellet and cytosol by centrifugation at 105,000 ×*g* for 30 min at 4 °C, using a Beckman LE-80K ultracentrifuge [20–22]. Cytosols were stored at −80 °C for future use.

Preparation of nuclear extracts

Reference powders were prepared from either de-identified human breast carcinomas or from uterine tissue specimens and homogenized using a Polytron PT 10–35 in 10 mM HEPES buffer, pH 7.9 containing 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.01 μg/μL aprotinin, leupeptin and pepstatin A. These reference powders were prepared previously as standards for assessment of estrogen and progestin receptor activities used in clinical trials for human breast cancer such as those conducted by the NSABP [8,23]. Lysates were centrifuged at 4 °C at 15,000 ×*g* for 10 min. Each nuclear pellet was resuspended in 20 mM HEPES buffer, pH 7.9 containing 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, and 0.01 μg/μL aprotinin, leupeptin and pepstatin A. The resuspended pellets were mixed at 4 °C for 2 h and centrifuged 4 °C, 15,000× g for 10 min. Nuclear extracts were concentrated and desalted with Amicon Ultra YM-10 columns (Millipore). To enrich the extracts for ERE-BP, the twelve most abundant plasma proteins were depleted using a Seppro™ MIXED12 Kit (GenWay Biotech).

Labeling of ERE sequences

For labeling with [32_P]dATP, 30 μM synthetic double-stranded ERE sequences from the VitA2 gene (5′-GTCCAAAGTCAGGTCACAGTGAC CTGATC-3′) (Integrated DNA Technologies, Coralville, IA, www.idtdna.com) were incubated with Klenow DNA buffer (Promega, Madison, WI, www.promega.com), 5% β-mercaptoethanol (Sigma, St. Louis, MO, www.sigmaaldrich.com), 750 μM dCTP, dGTP, and dTTP (Promega, Madison, WI, www.promega.com), 1.25 μM [32_P]dATP (Perkin Elmer, Waltham, MA, www.perkinelmer.com) and 5 units DNA Polymerase I Large (Klenow) fragment (Promega, Madison, WI, www.promega.com) at 37 °C for 30 min, followed by a gravity-flow separation through a NICK column (GE Healthcare, Piscataway, NJ, www.lifesciences.com). The sample was eluted with 3 mL TE buffer (10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA) and collected into fractions (~100 μL each). Radioactivity was measured by a scintillation counter and fractions were also run on a 4% polyacrylamide gel to confirm the presence and separation of labeled ERE from unreacted [32_P]dATP [18].

Electrophoretic mobility shift and supershift assays

Protein extracts were incubated with 50 ng non-specific DNA, poly (dI–dC) (GE Healthcare, Piscataway, NJ, www.lifesciences.com), 10 mM KCl, 1% glycerol and [32_P]-labeled VitA2–ERE sequences in 40 mM Tris–HCl buffer, pH 8.0, containing 500 μM PMSF and 10 μM monothioglycerol overnight at 4 °C. The sequence of the VitA2–ERE is 5′-GTCCAAAGTCAGGTCACAGTGACCTGATC-3′ [24]. For supershift assays, extracts were incubated with various antibodies for 30 min before the addition of [32_P]ERE. ERα antibodies used included AER 314, 320, 611, 1D5, 5D11 (Lab Vision/Thermo Fisher Scientific, Kalamazoo, MA, www.labvision.com) and ERα pAb (PanVera/Life Technologies, Grand Island, NY, www.invitrogen.com). ERβ antibodies used included Ab-15 (Lab Vision/Thermo Fisher Scientific, Kalamazoo, MA, www.labvision.com), MA1-310, PA1-310, PA1-311 and PA1-313 (Affinity Bioreagents/Thermo Fisher Scientific, Rockford, IL, www.pierce-antibodies.com). Additional antibodies used were the following: Ku70/Ku80 (3F247, monoclonal Santa Cruz), Ku70/80 (Ab-3, monoclonal, Thermo Fisher Scientific), Ku80 (Ab-2, monoclonal, Thermo Fisher Scientific) and Ku80 (C48E7, monoclonal, Cell Signaling). Reactions were separated by 5% polyacrylamide gel electrophoresis. Each gel was pre-run for 30 min at 150 V at 4 °C in 0.5× TBE buffer (1.0 M Tris–HCl, pH 8.6, containing 831 mM boric acid, 10 mM EDTA). Samples were then loaded, followed by electrophoresis at 300 V for 5 min, and then 180 V for 4 h. Subsequently, gels were dried and exposed to phosphor screens (Perkin Elmer, Waltham, MA, www.perkinelmer.com) overnight. The bands representing [32_P]ERE–protein complexes and free [32_P]ERE were visualized and quantified using a Cyclone Storage Phosphor System with OptiQuant® software (Perkin Elmer, Waltham, MA, www.perkinelmer.com).

Technique for determining DNA-binding activities

DNA binding activity levels of ERE-BP in breast cancer biopsies were determined by measuring the band intensity of either the ERE-BP/ERE complexes or free [32_P]ERE in each lane from the EMSA using OptiQuant® software. The value of band intensities (representing the amount of [32_P]Vita2–ERE) is reported as digital light units (DLU)/μg protein and normalized to the total DLU of the lane in order to compare between samples. A representative band quantification is shown in Fig. A.1A & B. Fig. A.1C illustrates that measurements of the DNA-binding activities are taken in the linear range of detection.

Purification of ERE-BP

Depleted nuclear extracts (800 μg of total protein) were incubated overnight at 4 °C with 300 pmol of either a biotinylated or unlabeled VitA2 ERE sequence in 20 mM HEPES buffer, pH 7.9 containing 800 ng of poly (dI–dC) (Amersham), 10 mM KCl and 1% glycerol. Candidate ERE-BP/ERE complexes were then incubated with 300 μL of NeutrAvidin agarose beads (Pierce) for 4 h at 4 °C with constant