Detection of the Level of Estrogen Receptor and Functional Variants in Human Breast Cancers by Novel Assays

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ABSTRACT

The level of estrogen receptor (ER) is a key determinant for the management of ER-positive [ER(+)] breast cancer patients. Growth of many human breast cancers is regulated by estrogen (E2) and progesterone (Pr). Generally, the ER in ER(+) breast cancer patients is targeted for therapy with antihormones. However 40% of ER(+) patients do not respond to antihormone therapy. Thus, the identification of antihormone resistant ER(+) breast cancers is essential for therapeutic predictions. Although ³H-E2 binding and immunodetection can identify ER, these procedures do not assess the functional state of the receptor molecule. In this study we describe a novel and rapid assay for the detection of ER and its functional state on the basis of the downstream interaction with its response element (ERE) based on the preferential binding of DNA-protein complex (ERE-ER) to a nitrocellulose membrane (NMBA). This method permits measurement of both the total and the functional fraction of ER. The ER status was examined in breast cancer cell lines and in breast cancer biopsy specimens by (i) ³H-E2 binding assay, (ii) immunodetection assays and (iii) by its interaction with ³²P-ERE. The sensitive NMBA assay was validated with well-characterized ER(+) breast cancer cell lines and also identified functional variants of ER among breast tumor biopsy specimens.

INTRODUCTION

Estrogen (E2)-regulated growth is the basis of anti-estrogen-targeted therapy for many human breast cancer patients (10–12,18). The level of estrogen receptor (ER) is a primary determinant for ER-targeted therapy. This is based on the mode of action of the steroid family of receptors including ER, which are hormone-activated transcription factors. ER is present in cells as an inactive complex associated with the inhibitory heat shock protein hsp90 (20). Binding of E2 to the receptor releases this protein and initiates a series of downstream events (14,21) resulting in overexpression of genes responsible for enhanced and uncontrolled growth of breast cancer cells (6,14,19).

Compounds such as tamoxifen (TAM) also bind to ER but cannot confer the active configuration to the receptor in mammary epithelial cells, thereby blocking the subsequent events (9,12,13,16,19). These compounds are designated as antihormones and are candidates for breast cancer therapy. Their therapeutic activity is limited to ER-positive [ER(+)] breast cancers. However, only 60% of ER(+) breast cancer patients respond to TAM and other antihormone treatments. The remaining ER(+) and all ER-negative [ER(-)] breast cancers constitute a major fraction that does not respond to antihormone therapy.

On the basis of the level of ER and its functional state, human breast cancer cells can be classified into three broad classes: Class 1 breast cancer cells (e.g., MCF-7 and T47D) are ER(+), hormone dependent and responsive. Patients with this class of cells are successfully treated with antihormones.

Class 2 cells (e.g., 21PT) are ER(+), hormone independent and nonresponsive (3). As this class of breast cancer cells is resistant to antihormones, these breast cancer patients cannot be treated with antihormones and are subjected to combination chemotherapy. The Class 3 breast cancer cells (e.g., MDA-MB-231 and MDA-MB-435) are ER(-), hormone independent, hormone nonresponsive and antihormone resistant.

To determine the feasibility of antihormone therapy, these three classes of breast cancers need to be experimentally distinguished from each other. The classical radioactive hormone binding or immunodetection assays for ER cannot differentiate between the mutated ER(+) breast cancers and functional variants with structural alterations of ER (3). To distinguish between these two classes of ER(+) breast cancers, we have developed an alternative downstream functional assay based on its interaction with response element (ERE) and the DNA-protein complex formation (ERE-ER), which preferentially binds to nitrocellulose membrane. By using this approach we have identified ER variants among established breast cancer cell lines and human breast cancer biopsy specimens. This assay could subclassify human breast cancers on the basis of the functional state of ER and predict appropriate therapy for different types of breast cancer patients.

MATERIALS AND METHODS

Cell Lines

The ER(+) MCF-7 and T47D and ER(-) MDA-MB-231 cell lines were obtained from ATCC (Manassas, VA,

USA). The 21PT cell line was established from a primary breast tumor by Sager and colleagues (17,22). 21PT was initially described as ER(-) based on a ligand binding assay (17,22). ER-specific mRNA, ER protein and the E2-responsive gene product PR were subsequently detected in 21PT cells (3). This cell line, therefore, is redesignated as a hormone-independent subclass of ER(+) cells (3). Breast cancer cells were maintained in stock medium [DMEM supplemented with 10% fetal bovine serum (FBS) and 2.8 μ M hydrocortisone, 1 μ g/mL insulin and 12.5 ng/mL epidermal growth factor (EGF) and an-

tibiotics] at 37°C in 5% CO₂ and 95% air. The rich medium (R) is DMEM supplemented with 10% FBS without the growth factors. The basal medium (B) is DMEM supplemented with 10% dextran-coated charcoal-treated FBS (Hyclone Laboratories, Logan, UT, USA).

Materials

Monoclonal anti-ER-antibody TE1-11, raised against the C-terminal amino acid residues 300–595 of human ER, was obtained from NeoMarkers (Fremont, CA, USA). Purified human re-

combinant ER was purchased from PanVera (Madison, WI, USA). The Hybond® nitrocellulose membrane and ECL® immunodetection kit were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Hydroxylapatite was obtained from Calbiochem-Novabiochem (San Diego, CA, USA). ³H-E2 [2,4,6,7-³H (N) Estradiol; 72 Ci/mmol] was purchased from NEN Life Science Products (Boston, MA, USA). Synthetic single-stranded complementary oligonucleotides containing a wild-type ERE motif (5'-GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3') was obtained from Integrated DNA

Figure 1. Detection of ³²P-ERE-ER complex by NMBA and EMSA in human breast cancer cell lines. MDA-MB-231, MCF-7, T47D and 21PT cells were maintained in stock medium, and the experiments were carried out by growing the cells either in rich (R), basal (B) or basal medium supplemented with E2 (10⁻⁶ M for 2 h). Nuclear extracts (8) were prepared, and protein content (5) was measured as described in Materials and Methods. Duplicate reactions with 5 μ g nuclear proteins were incubated under mobility shift assay conditions as described previously (3,14). Half of the reaction was analyzed by NMBA (A) in a dot blot assembly system, and the other half was analyzed by EMSA (B) as described in Materials and Methods. The membrane was air-dried and exposed to X-ray film for autoradiography (A), and the gel was dried under vacuum and also exposed to X-ray film for autoradiography (B).

Figure 2. Nitrocellulose filter binding assay for the measurement of DNA bound and total ER in breast cancer cell lines. MDA-MB-231, MCF-7, T47D and 21PT cells were grown in rich medium under standard tissue culture conditions. To one set (A) 10 ng double-stranded nonradioactive ERE and 10 μ g nuclear extract protein were added in the reaction mixture. "None" contained 10 μ g BSA in place of nuclear extract. Samples in duplicate were applied to the membrane, washed with PBS containing 0.2% Tween® 20 and 5% dried milk and incubated with antihuman ER-antibody (10 ng/10 mL, TE1-11) for 1 h at room temperature with gentle rocking. The membrane was then washed with PBS-Tween 20 solution, incubated with secondary anti-mouse IgG-HRP conjugate in Blotto solution (1:1000 dilution) and subjected to the ECL immunodetection system. The second set of reactions (B) contained in 10 μ L, 10 μ g nuclear extract and ³²P-ERE-oligonucleotide (10 ng, approximately 30000 cpm) and were incubated for 30 min at room temperature. Reaction sample 5 μ L was analyzed by EMSA, and another 5 μ L was applied to a nitrocellulose membrane assembled in a multiwell slot system and processed as above. The dried membrane was then exposed to X-ray film for autoradiography. EMSA analyses (C) of 5- μ L aliquots in duplicate of reactions, on 6% nondenaturing PAGE, showed a similar pattern of ERE/ER signals as in panels A and B. These experiments were repeated three times, and similar results were obtained.

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Technologies (Coralville, IA, USA). The complementary strands were annealed to generate the double-stranded ERE oligonucleotide. 17β -Estradiol (E2), hydrocortisone, insulin, dithiothreitol, dimethyl sulfoxide and phenylmethylsulfonyl fluoride were obtained from Sigma (St. Louis, MO, USA).

Preparation of Cell and Tissue Extracts

Nuclear extracts from cultured cells were prepared as described previously (2,4), following the procedure described by Dignam et al. (8).

Frozen human breast tumor specimens were thawed slowly, and associat-

ed fibrous and nontumor tissues were removed with a sterile blade. The tissue was then minced and transferred to a tight Dounce homogenizer in an equal volume of buffer II (8) and homogenized with 30 strokes until all the soft tissues were pulverized. The homogenized suspension was then centrifuged at 800 rpm for 5 min in rotor J18 in a refrigerated centrifuge (Beckman Coulter, Fullerton, CA, USA). The supernatant was removed into another microcentrifuge tube and centrifuged at 10 000 rpm for 10 min. The supernatants were then dialyzed against a large volume of buffer III (8); the protein content was determined (5) and saved in small aliquots at -70°C .

Ligand Binding Assay

The ER(+) and ER(-) breast cancer cell lines were grown in E2-free and phenol red-free medium (B) for 48 h, and nuclear extracts were prepared (8). The ER level was determined in the nuclear extracts and quantitated by the hydroxylapatite method for ^3H -E2 binding as described by Obourn et al. (15). ER level was similarly determined in the extracts of frozen tissues prepared as described above.

Immunodetection of Total ER

This was accomplished by following the nitrocellulose membrane binding assay (NMBA) protocol. An aliquot of the nuclear, cellular or tissue extracts (10–50 μg protein) was incubated in the presence of nonradioactive ERE-oligonucleotide under conditions for the formation of ERE-ER complex in a 10–100 μL reaction mixture in each well of a 96-well plate. Details of the binding conditions are described previously (2,14). The reaction mixtures were then applied to a nitrocellulose membrane in a multislot blot system (Schleicher & Schuell, Keene, NH, USA) of the size of a 96-well cloning plate, assembled and prewashed with binding buffer (2,14). The samples were then adsorbed to the membrane for 15 min at room temperature, washed with binding buffer, reacted with specific primary antibody followed by secondary antibody conjugated with horseradish peroxidase (HRP) and finally subjected to an ECL immunodetection system. The immunodetection signals were quantitated by densitometric scanning followed by integration of the signals with the Multi-Analyst program version 1.0.2. (Amersham Pharmacia Biotech). ER levels are quantitated by extrapolation from a standard curve with purified ER generated under the identical experimental conditions.

Detection of ^{32}P -ERE-ER Complex by NMBA

Synthetic double-stranded oligonucleotides of ERE were end-labeled with [^{32}P]ATP in the presence of T4 polynucleotide kinase (1). Reactions containing 10–50 μg nuclear protein from cul-

Figure 3. ERE-ER interaction in human breast tumor biopsy specimens by NMBA and EMSA. Nuclear extracts from ER(+) breast cancer cell lines (MCF-7 and T47D) and tissue extracts from biopsy specimens (BTT1, invasive ductal/stage I, age 45; BTT2, ductal/lobular, stage II, age 76; BTT3, invasive ductal/stage II, age 56; and BTT4, invasive ductal, stage I, age 63) were prepared as described above. (A) ER status of these cell lines and biopsy specimens as determined in duplicate by ^3H -E2 binding assay following the protocol of Obourn et al. (15). The ^{32}P -ERE-ER complex in nuclear extracts (10 μg) and tissue extracts (20 μg) was determined by EMSA as described (20,21). (B) ^{32}P -ERE-ER complexes immobilized on nitrocellulose membrane and determined by NMBA. This experiment was repeated twice, and a similar pattern was observed in both experiments.

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tured cells or tissue extracts of biopsy specimens plus 10 ng ^{32}P -labeled (approximately 30 000 cpm) of ERE (shown above) were incubated for 30 min at room temperature. The reaction mixtures were then applied to a pre-washed nitrocellulose membrane assembled in the multislot blot system and treated and washed as described above. ^{32}P -ERE-ER complex was detected by autoradiography of the membrane and quantitated by densitometric scanning and integration. ER levels are quantitated by extrapolation from a standard curve with purified ER generated under the identical experimental conditions.

Electrophoretic Mobility Shift Assay

Reaction mixtures with ^{32}P -labeled double-stranded ERE plus nuclear ex-

tracts from cultured cells or tissue extracts of biopsy specimens and other components as described above were incubated at room temperature for 30 min. The reaction mixtures were then subjected to electrophoretic mobility shift assay (EMSA), and the DNA-protein complex was detected as a retarded radioactive band by autoradiography of the dried gel (2–4). The retarded ^{32}P -ERE-ER complex was characterized by (i) competition experiments with non-radioactive wild-type and mutant double-stranded ERE-oligonucleotide and (ii) by comparative direct binding studies with ^{32}P -labeled mutant and wild-type ERE-oligonucleotide as described previously (3). The mobility of ^{32}P -ERE-ER complex with pure recombinant ER and ^{32}P -ERE oligonucleotide was identical to the ones ob-

served with nuclear extracts from breast cancer cell lines and tissue extracts (2,3). The specificity of this DNA-protein interaction was established by carrying out a reaction with nuclear extract from ER-deficient MDA-MB-231 cells in the absence or presence of purified recombinant ER (15). The intensities of the autoradiographic signals were quantitated by scanning with a densitometer, and the results are presented as integrated intensity (arbitrary numbers).

RESULTS

Functional State of ER

The ER status in nuclear extracts of human breast cancer cell lines and biopsy specimens was studied by (i) ER binding of ^3H -E2, (ii) immunodetection of the receptor and (iii) its functional state by interaction with ^{32}P -ERE using EMSA and NMBA. A comparative analysis of ER levels in the nuclear extracts of four cell lines, ER(+) and hormone-dependent MCF-7 and T47D, ER(+) and hormone-independent 21PT and ER(-) MDA-MB231 cells (3), by these three methods generated similar results on the ER levels, and these are comparable to those reported in the literature (data not shown and References 17 and 22).

Classification of Breast Cancer Cell Lines by NMBA

Hormone action in ER(+) MCF-7 and T47D cells is dependent on the initial interaction of E2 with ER (3). The downstream events of ER interaction with ERE and transactivation of responsive promoter are also dependent on E2, thereby classifying these as E2-dependent ER(+) breast cancer cells. Although specific mRNA and protein is detected in 21PT breast cancer cells, ER in these cells does not bind to ^3H -E2 (3,17,22). The downstream events of interaction with ERE and transactivation of E2-responsive genes are independent of E2, thereby classifying 21PT as E2-independent ER(+) cells (3).

This distinction between these two classes was reexamined by our newly developed NMBA assay. Results of

Figure 4. Quantitation of DNA bound and total ER in breast tumor cells. (A) The standard curves generated with purified recombinant ER (fmol) by NMBA using ^{32}P -ERE and by immunodetection assay. The binding reaction mixture of 10 μL contained all the components and indicated amounts of purified ER and 5 μg nuclear extract protein of ER(-) MDA-MB231 cells. Other binding conditions are similar to those described in Materials and Methods. The autoradiographic signals generated by 5 μg MDA-MB231 nuclear extracts was insignificantly low as shown in Figure 2B. The nonspecific binding was determined by running the reaction in the presence of 100-fold excess of nonradioactive double-stranded ERE for each concentration of purified ER. The specific signal intensity was determined by deducting the signal intensity observed in the presence of excess nonradioactive ERE. A typical standard curve was also generated with purified ER by the immunodetection assay that is shown in panel A. Details of the immunodetection assay are described in Figure 2A. The intensities of the autoradiographic and immunoassay signals were quantitated by scanning with an imaging densitometer (Bio-Rad GS-700; Bio-Rad Laboratories, Hercules, CA, USA), followed by integration of the signals with the Multi-Analyst program version 1.0.2. Triplicate samples were run, and standard deviations are shown. (B) The ratio of the fraction of DNA bound ER determined by quantitation of the autoradiographic signals and total ER determined by quantitation of the immunodetection signals in the tissue extracts of 6 ER(+) human biopsy specimens. Tissue extracts were prepared as described in Methods and Materials. A larger reaction mixture of 100 μL containing 50 μg tissue extract protein and other components was incubated for 30 min at room temperature. The radioactive reaction mixtures for the determination of DNA bound fraction and the nonradioactive reaction mixtures for immunodetection assay of total ER were applied to separate membranes assembled in a multislot blot system and processed as described in Materials and Methods. The radioactive membrane was subjected to autoradiography, and the nonradioactive membrane was subjected to immunodetection with anti-ER antibody as described in the legend to Figure 2. The respective signals were quantitated by extrapolation from the standard curves generated under the identical assay conditions as described in panel A.

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Figure 1 verified their phenotypes. The low radioactive signal observed in the nuclear extracts of MDA-MB231 cells grown either in rich medium, basal medium or in the presence of E2 was attributed to nonspecific binding (Figure 1A, row 1, lanes 1–6) and was comparable to that observed with the same amount of BSA (data not shown), thereby confirming the ER(-) phenotype of these cells. The ^{32}P -ERE binding with ER in the nuclear extracts was dependent on the treatment of ER(+) MCF-7 and T47D cells with E2 (Figure 1A, rows 2 and 3, lanes 1–6). The ^{32}P -ERE-ER complex formation was detected in the nuclear extracts of both E2-untreated and treated 21PT cells (Figure 1A, row 4, lanes 1–6), thereby reestablishing the E2-independent phenotype of 21PT cells by NMBA (3). These properties of the two classes of ER(+) breast cancer cells were reaffirmed by analyzing part of the reaction mixture by EMSA (Figure 1B) and validated the NMBA assay.

Measurement of Total and DNA-Bound Fraction of ER (ERE-ER) by NMBA

Total ER-protein can be detected with an anti-ER antibody followed by immunodetection with the ECL system because both free ER and DNA-bound fraction are immobilized on the nitrocellulose membrane. Nuclear proteins from three ER(+) breast cancer cell lines were immobilized on the nitrocellulose membrane, and ER was immunodetected with anti-ER antibody (Figure 2A). Reactions containing no extracts (Figure 2A, row 1) or extracts from ER(-) MDA-MB-231 cells (Figure 2A, row 2) generated no signals. In contrast, strong signals were detected in nuclear extract (duplicate) from ER(+) MCF-7 (Figure 2A, row 3), T47D (Figure 2A, row 4) and 21PT (Figure 2A, row 5) cells by immunodetection on the nitrocellulose membrane. These results provided information on the total ER as determined by immunodetection with NMBA.

The functional fraction of ER in the nuclear extracts of the cell lines was determined by NMBA. The autoradiographic signals on a nitrocellulose membrane obtained from the reactions

containing ^{32}P -labeled ERE-oligonucleotide represent immobilized ^{32}P -ERE-ER complex (Figure 2B). Free ^{32}P -ERE-oligonucleotide DNA was found to bind very weakly (Figure 2B, row 1). A reaction containing nuclear extract of ER(-) MDA-MB-231 in the presence of ^{32}P -ERE-oligonucleotide also showed insignificant binding to the nitrocellulose membrane (Figure 2B, row 2). However, nuclear extracts from ER(+) breast cancer cell lines MCF-7 (Figure 2B, row 3), T47D (Figure 2B, row 4) and 21PT (Figure 2B, row 5) formed ^{32}P -ERE-ER complexes as indicated by the strong positive autoradiographic signals. The immunodetection (Figure 2A) assays carried out with nonradioactive ERE-oligonucleotide gave a qualitatively similar pattern of signals as obtained with ^{32}P -ERE-oligonucleotide (Figure 2B). Results of the autoradiographic detection by NMBA of ^{32}P -ERE-protein complex (Figure 2B) were similar to those obtained by the classical detection method for DNA-protein complex, EMSA (Figure 2C). Thus, the simplified NMBA was able to replace the complicated EMSA that involves lengthy procedures of electrophoresis and autoradiography of the dried gel for measurement of ERE-ER interaction. Furthermore, immunodetection signals (Figure 2A) represent the total ER, whereas the autoradiographic signals in Figure 2B represent the ER that is bound to ERE. Thus, NMBA assays provide information on the functional fraction of the total ER.

ER Status in Tumor Tissue Specimens

Initially, we studied ER properties of four tumor biopsies. On the basis of ^3H -E2 binding, ER in two of these tumor tissues (BTT1, invasive ductal/stage I and BTT2, ductal/lobular, stage II) are designated as ER(+), like MCF-7 and T47D cells. The other two (BTT3, invasive ductal/stage II and BTT4, invasive ductal/stage I) did not bind E2 and are classified as ER(-). We determined ERE-ER complex formation in the nuclear extracts of these biopsy specimens by EMSA (Figure 3A). ^{32}P -ERE-ER complexes of similar size to those observed in nuclear ex-

tracts of ER(+) MCF-7 and T47D cells were observed in the ER(+) (BTT1 and BTT2) breast tumor tissue extracts. As expected, an ERE-ER complex was not detected in the nuclear extract of one of the ER(-) breast tumor tissue (BTT3). However, the ER in BTT4 that did not bind to E2 did interact with ERE independently of hormone binding. The ER variant in this breast tumor specimen is similar to the class identified in 21PT cells. We next compared NMBA results (Figure 3B) with those obtained by EMSA (Figure 3A). Both methods provide the same conclusions, establishing the validity of NMBA.

Measurement of DNA-Bound Fraction and Total ER in Breast Cancer Biopsy Specimens by NMBA

We have measured ER level in the extracts of six breast tumor biopsy specimens that are designated by the clinical laboratory as ER(+) on the basis of the ^3H -E2 binding assay. We reclassified these biopsy specimens by the two NMBA assays, one of which determined the DNA-bound fraction (^{32}P -ERE-ER complex) and the second determined the total ER by immunodetection of the receptor protein immobilized on the membrane. Both autoradiographic and immunodetection signals were quantitated by densitometry and extrapolation from the linear region of the simultaneously run standard curves with purified ER. Typical standard curves for ^{32}P -ERE-ER complex formation and immunodetectable total ER in the presence of the indicated amounts of purified recombinant ER are shown in Figure 4A. Results obtained with triplicate samples demonstrate that the sensitivity of the assay in the lower range is between 5 and 10 fmol ER in the reaction mixture. The volume of the reaction mixture can be varied from 10 to 100 μL to accommodate larger amounts of extract protein of samples with low ER levels.

The ^{32}P -ERE-ER complex measured by NMBA reflects the level of DNA-bound fraction, whereas immunodetection measures the total ER in the same sample. Results of Figure 4B show the ratio between the ERE-bound fraction and the total ER in the extracts

of these six different biopsy samples. ER levels in three samples (Figure 4B, samples 3, 4 and 5) show a ratio close to 1, suggesting that most of the ER fraction in these tumors is bound to DNA. DNA-bound ER in two other samples (Figure 4, samples 1 and 2) is lower than 1, suggesting that only a fraction of the total ER is bound to ERE. The higher ratio (more than 1) between the DNA bound and total ER observed in the extract of sample 6 remains to be clarified. This type of comparative analysis thus provides information on the functional state and allows subclassification of ER in breast tumor biopsy specimens.

DISCUSSION

In this study, we report a useful assay to distinguish ER-related phenotypes of different classes of breast can-

cers. We investigated breast cancer cells in culture as models and then proceeded to biopsy specimens. The success of this strategy in identifying ER variants is dependent on the determination of the level and functional state of ER. It could allow therapeutic predictions and could avoid failures of target-directed therapy for ER(+) antihormone-resistant breast cancer patients.

The functional criterion chosen in this study is ERE-ER complex formation, a downstream event of E2 action that follows the initial interaction of the hormone with its specific receptor ER. We have established a simplified assay for identifying this DNA-protein complex, by a procedure that is not as complicated as the classical DNA-protein interaction assay, EMSA. This new procedure (NMBA) is based on the preferential binding of the DNA-protein complex to nitrocellulose membrane versus the unbound free DNA (1). It is

applicable not only to established cell lines but also to human breast tumor biopsy specimens. Simultaneous analysis by NMBA and EMSA generated comparable results, establishing the validity of this new method for determining the functional state of ER. By carrying out both immunodetection and radioactive NMBA assays, the fraction of the total ER that is functional in breast tumor biopsy specimens is determined.

The significance and advantages of this method over the current methods for detection of ER in human breast cancer biopsy specimens are (i) the ability to determine the functional state of ER rather than total protein as monitored by the current immunostaining or ligand binding assays; (ii) the ability to make therapeutic and prognostic predictions, specifically for antihormone-resistant breast cancer patients; (iii) the ability to handle a large number of samples; (iv) its high sensitivity; and (v) the

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involvement of very few steps. For clinical applications, radioactive ERE could be replaced with a fluorescent tag or with a biotinylated ERE-oligonucleotide followed by amplification of the signals with streptavidin-phycoerythrin fluorescent conjugate. However, the sensitivity of these methods has to be improved to the level of NMBA using ^{32}P -oligonucleotide.

These results suggest that NMBA is applicable for the determination of other DNA-protein complexes such as progesterone response element-progesterone receptor (PRE-PR) and may, in general, be used for the determination of the functional state of transcription factors such as nuclear factor kappa B (NF- κ B). We have extended NMBA successfully for the determination of PR in extracts of cultured cells and tissue specimens (data not shown). The level of immunoreactive PR is used routinely as a clinical marker for ER function. But PR synthesis can occasionally be independent of ER. Thus, PR level may not always reflect the functional state of ER (7).

Assays such as NMBA, based on ERE-ER interaction, provides an exact state of downstream functions of the receptor. The breast cancer patients can be divided into two broad classes, ER(+) and ER(-). Antihormone therapy is applicable only to ER(+) patients. It is not, however, applicable to all ER(+) breast cancer patients in which ER status is determined based only on the ligand binding and immunodetection assay. In a subclass of ER(+) breast cancer cells, modified ER may not bind to ERE, although it may bind to E2 and be detectable by immunoassay. This class of ER(+) patients should be resistant to antihormones. Such therapeutic predictions can only be made on the basis of ERE-ER interaction measured by NMBA assay (data not shown). The subclass of ER(+) cells, such as 21PT, that is misclassified as ER(-) because of the lack of E2 binding should also be resistant to antihormone therapy. Although the ER protein was detected in 21PT cells by immunoassay, its functional state, such as ERE-ER complex formation, could only be assessed by the NMBA assay. However, these antihormone-resistant cells were sensitive to an alternative drug, a calmodulin in-

hibitor (W7) (4). Predictions for such alternative therapy for these patients could be made on the basis of determining the functional status of ER by the NMBA assay. Thus, using the NMBA assay on the extracts of biopsy specimens allows one to propose alternative potential targets and treatment protocols or therapy of TAM-resistant ER(+) breast cancer patients.

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