Abstract

**Purpose:** In addition to genomic signaling, it is accepted that estrogen receptor-α (ERα) has nonnuclear signaling functions, which correlate with tamoxifen resistance in preclinical models. However, evidence for cytoplasmic ER localization in human breast tumors is less established. We sought to determine the presence and implications of nonnuclear ER in clinical specimens.

**Experimental Design:** A panel of ERα-specific antibodies (SP1, MC20, F10, 60c, and 1D5) was validated by Western blot and quantitative immunofluorescent (QIF) analysis of cell lines and patient controls. Then eight retrospective cohorts collected on tissue microarrays were assessed for cytoplasmic ER. Four cohorts were from Yale (YIMA 49, 107, 130, and 128) and four others (NCI YIMA 99, South Swedish Breast Cancer Group SBII, NSABP B14, and a Vietnamese Cohort) from other sites around the world.

**Results:** Four of the antibodies specifically recognized ER by Western and QIF analysis, showed linear increases in amounts of ER in cell line series with progressively increasing ER, and the antibodies were reproducible on YTMA 49 with Pearson correlations ($r^2$ values) ranging from 0.87 to 0.94. One antibody with striking cytoplasmic staining (MC20) failed validation. We found evidence for specific cytoplasmic staining with the other four antibodies across eight cohorts. The average incidence was 1.5%, ranging from 0 to 3.2%.

**Conclusions:** Our data show ERα is present in the cytoplasm in a number of cases using multiple antibodies while reinforcing the importance of antibody validation. In nearly 3,200 cases, cytoplasmic ER is present at very low incidence, suggesting its measurement is unlikely to be of routine clinical value. *Clin Cancer Res; 18(1); 118–26. ©2011 AACR.*

Introduction

The estrogen receptor (ER) is the oldest and most successful biomarker that exists in breast cancer today (1–3); however, roughly 50% of ER-positive patients still exhibit de novo or acquired resistance to tamoxifen, suggesting that more complex mechanisms are operating in these patients (4). In addition to the classical view of the ER as a nuclear hormone receptor, in the past 10 years, it has become accepted that ER-alpha has nonnuclear signaling functions, referred to as nongenomic signaling. In the case of breast cancer, this nongenomic signaling can involve full-length receptor or other isoforms (5–9), as well as cross talk with other growth factor receptors (GFR; refs. 4, 10–12) or cytoplasmic kinases such as Src (13–15). In preclinical models, nongenomic signaling has been shown to underlie tamoxifen resistance (13, 16–20).

The current guidelines for measuring ER in a clinical setting, however, assess only nuclear staining using immunohistochemistry (IHC; ref. 21). The presence of cytoplasmic or membranous immunoreactivity is ignored or assumed to be nonspecific, and while individual pathologists may observe it from time to time, there is no available record of the incidence of such staining. The few reports in literature are all in cell line models, and none have shown concrete evidence to date of any cytoplasmic ER in actual breast cancer cases. Furthermore, some of these studies have used antibodies with less rigorous validation. We have previously found antibody validation to play a critical role in evaluation of protein localization (22), and have thus developed extensive antibody validation protocols (23). We also have established the use of quantitative immunofluorescence (QIF), commercialized as AQUA technology (HistoRx Inc.), to assess expression and localization of a wide range of biomarkers on tissue microarrays (TMA;
Clinicians today rely on measurement of nuclear estrogen receptor (ER) as a hallmark of breast cancer diagnosis and treatment. However, for over 10 years it has been widely accepted that ER exists and functions outside the nucleus, contributing to growth and survival as well as resistance to endocrine therapies in cellular models, suggesting its measurement could be of prognostic or predictive value, and could help address the problem of tamoxifen resistance. However, in the first study to thoroughly examine the incidence of cytoplasmic ER in human cases, we find an incidence of only 1.5%, suggesting cytoplasmic ER may only be a transient occurrence or an artifact of immunostaining, and is therefore unlikely to be of predictive value in the current clinical setting.

**Translational Relevance**

In total, 8 different cohorts of archival breast cancer cases, present on formalin-fixed paraffin-embedded TMAs, were examined for evidence of cytoplasmic ER by immunofluorescence. One of the cohorts (YIMA 49) was also examined by traditional IHC. Four of the TMAs were constructed at Yale according to previously published guidelines (31): YIMA 49 (diagnosed: 1962–1982, n = 619, stained with 1D5, 60c, SP1, and F10), YIMA 107 (collaboration with University of Michigan as part of a tamoxifen pharmacogenetics clinical trial (32), n = 179, stained with 1D5), YIMA 130 (diagnosed: 1976–2005, n = 526, stained with 1D5 and SP1), and YTIMA 128 (diagnosed: 2003–2006, n = 257, stained with 1D5 and SP1). Four other cohorts came from outside institutions: NCI YTMA 99 (a Polish multi-institutional trial, n = 732 with triplicate cores for each case, stained with 1D5), NSABP B14 [a multi-institutional trial (33), n = 956, stained with SP1], South Swedish Breast Cancer Group SBII (34, 35; n = 556, stained with 1D5), and a Vietnamese Oophorectomy cohort (36) for more information, n = 156, stained with SP1).

**Index TMA**

Whole-cell lysates were prepared in buffer containing 1% Nonidet P-40, 20 nmol/L Tris-HCl pH 8.0, 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L NaVO₄, and complete mini EDTA-free protease inhibitor cocktail (Roche) in dH₂O. Twenty-five micrograms of each lysate was resolved by SDS-PAGE on a 4% to 12% Bis-Tris gel (NuPage), using NuPage MOPS SDS Running Buffer at 45 mA. Resolved protein was transferred using NuPage Transfer Buffer at 50 V for 2 hours. Western blot analysis was carried out according to standard procedures, using the ER antibodies above.

**Materials and Methods**

**Cell culture**

A panel of ATCC breast cancer cell lines was chosen to span a range of ER expression. In addition, MCF-7 cells engineered with doxycycline-inducible ER overexpression were used, which were a gift from Elaine Alarid [see Fowler and colleagues (28)]. All cells were maintained at 37°C and 5% CO₂, and grown either in suggested media, or in RPMI-1640 culture medium (Gibco) supplemented with 10% FBS (Gemini BioProducts), 100 units/mL penicillin G and 100 μg/mL streptomycin (Gibco), 1 mmol/L sodium pyruvate (Gibco), and 2 mmol/L L-glutamine (Gibco).

**Antibodies**

Antibodies were selected for validation based on previous use in the literature. The 5 antibodies selected were F10 (Santa Cruz) used at 1:1,000 overnight at 4°C; SP1 (Thermo) used at 1:500 overnight at 4°C, 60c (Upstate) used at 1:2,000 overnight at 4°C, 1D5 (Dako) at 1:500 overnight at 4°C, and MC20 (Santa Cruz) at 1:500 for 1 hour at room temperature (RT). β-tubulin (Cell Signaling Technology, 2146), diluted 1:4,000, was used as a loading control for Western blot analysis. Rabbit and mouse cytokeratin antibodies (Dako) were used at 1:100 for immunostaining.

**Western blot analysis**

Whole-cell lysates were prepared in buffer containing 1% Nonidet P-40, 20 nmol/L Tris-HCl pH 8.0, 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L NaVO₄, and complete mini EDTA-free protease inhibitor cocktail (Roche) in dH₂O. Twenty-five micrograms of each lysate was resolved by SDS-PAGE on a 4% to 12% Bis-Tris gel (NuPage), using NuPage MOPS SDS Running Buffer at 45 mA. Resolved protein was transferred using NuPage Transfer Buffer at 50 V for 2 hours. Western blot analysis was carried out according to standard procedures, using the ER antibodies above.

**Immunofluorescent staining**

Slides were deparaffinized by melting at 60°C for 20 minutes, followed by soaking twice for 20 minutes in xylene (JT Baker). Rehydration was carried out twice in 100% EtOH for 1 minute, followed by 70% EtOH for 1 minute, and tap water for 5 minutes. Antigen retrieval was carried out in

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citrate buffer (3.84 g sodium citrate dihydrate in 2 L ddH2O, brought to pH 6.0 with 1 mol/L citric acid) using the PT module from LabVision. Endogenous peroxidases were blocked by 30 minutes of incubation in 2.5% hydrogen peroxide in methanol at RT. After washing, nonspecific antigens were blocked by incubation in 0.3% bovine serum albumin in TBST for 30 minutes at RT in a humidity chamber. Rabbit Cytokeratin (Dako), diluted 1:100 in block (BSA in TBST above), was incubated overnight at 4°C. ER was stained using various antibodies (noted under the Patient Cohorts section), which included 1D5 (Dako, 1:50 in block, incubated 1 hour at RT), SP1 (Thermo, 1:1,000 in block, incubated overnight at 4°C), F10 (Santa Cruz, 1:5,000 in block overnight at 4°C), 60c (Upstate, 1:5,000 in block overnight at 4°C), and MC20 (1:100 in block overnight at 4°C). Primary antibodies were followed by Alexa 546–conjugated Goat anti-Rabbit or anti-Mouse secondary antibody (Molecular Probes) diluted 1:100 in mouse or rabbit EnVision reagent (Dako) for 1 hour at RT. Signal was amplified using Cyanine 5 (Cy5)-tyramide (PerkinElmer) at a dilution of 1:50 for 10 minutes at RT. Nuclei were stained using 10 μg/ml DAPI (Molecular Probes) in block for 20 minutes at RT, and coverslips mounted with ProLong mounting medium (ProLong Gold, Molecular Probes).

**AQUA analysis**

ER immunofluorescence was quantified using AQUA. Briefly, a series of high-resolution monochromatic images were captured by the PM-2000 microscope (HistoRx) using AQUAitition 2.2 software (HistoRx). Images were collected for each histospot after autofocus and autoexposure. Fluorophores included DAPI (to create nuclear compartment), Cy3 (Alexa 546-cytokeratin to distinguish tumor from stroma and create cytoplasmic compartment), and Cy5 for the target (ER). Image analysis was carried out using AQUAlysis 2.2 software (HistoRx), which binarizes the cytokeratin stain (each pixel being "on" or "off") to create an epithelial tumor "mask." It uses a clustering algorithm to assign each pixel, with 95% confidence, to either a nuclear or cytoplasmic compartment. The AQUA score of ER in each subcellular compartment (nuclear, cytoplasmic, and whole tumor mask) is calculated by dividing the ER pixel intensities by the area of the compartment within which they were measured. AQUA scores are normalized to the exposure time, bit depth, and lamp hours at which the images were captured, allowing scores collected at different exposure times to be directly comparable.

**Immunohistochemical staining**

Immunohistochemical staining was carried out on YTMA 49 in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory, using either the 1D5 (Dako) or SP1 (Ventana) staining system for ER in accordance with manufacturer’s instructions. Slides were digitally scanned using the Biolimagene slide scanner and visually assessed using ImageViewer software (Biolimagene).

**Assessment of cytoplasmic ER**

In order for a case to be considered positive for cytoplasmic ER, the following conditions had to be met: (i) immunoreactivity was observed using 1 of 4 valid monoclonal antibodies (1D5, SP1, 60c, and F10), (ii) immunoreactivity colocalized with cytokeratin but did not colocalize with DAPI, (iii) immunoreactivity was robust (at least 25% the intensity of nuclear staining, or greater than nuclear staining), (iv) immunoreactivity was not due to out-of-focus tissue or bleed-through from any other channel, (v) immunoreactivity was observed on the same slide as a positive (nuclear staining with no background) and negative (no staining) control cases. For each cytoplasmic case, conditions 1 to 5 were confirmed by 2 separate individuals (A.W. Welsh and D.L. Rimm), including a certified pathologist (D.L. Rimm).

**Statistical analysis**

All statistical analyses (bivariate regressions) were carried out using StatView analysis software (SAS Institute Inc.).

**Results**

**Antibodies to multiple epitopes of ER are highly reproducible**

To see assess the existence of cytoplasmic ER in breast cancer cases, we first validated a panel of 5 antibodies raised against different epitopes of ER. We chose 5 antibodies previously reported in the literature to be specific and/or to detect cytoplasmic ER (see Fig. 1A). These included 1D5 (an N-terminal mouse monoclonal from Dako, the clinical standard), SP1 [a C-terminal rabbit monoclonal, reported to be more sensitive than 1D5 and less sensitive to signal degradation due to delays in fixation (37–40)], 60c [an N-terminal rabbit monoclonal from Upstate], F10 (a C-terminal mouse monoclonal we had previously validated in the lab), and MC20 [a rabbit polyclonal reported to show cytoplasmic ER staining (41–48)]. First, a panel of cell lines known with known ER status was examined by Western blot analysis. SP1, 60c, and F10 showed specific detection of the expected 66 kD band for full-length ER (Fig. 1B) in the 4 lines known to be ER-positive (BT474, MCF-7, T47D, and ZR751). These 4 antibodies (1D5, F10, SP1, and 60c) were then used to stain a large retrospective cohort of breast cancer cases from Yale (YTMA 49, diagnosed. 1962–1982) by QIF, and levels of nuclear ER were quantified using AQUA. All antibodies showed strong reproducibility on these 619 cases (Fig. 1C–H), with Pearson r2 values ranging from 0.87 to 0.94 (r values ranging from 0.93 to 0.97).

The next step in our antibody validation protocol is titration and analysis of specificity and reproducibility by immunofluorescence. Each cell line in the panel was formalin fixed, paraffin embedded, and cored as tissue blocks to create the index TMA, which also contained a panel of 40 breast cancer control cases in duplicate (spanning the range of ER expression). After titration on breast test TMAs to optimize their dilution, each antibody was individually used to stain the index TMA by QIF. Specific nuclear staining
was observed in the 4 ER-positive cell lines using 1D5, SP1, 60c, and F10. Furthermore, progressively increased staining was seen in a cell line series induced to gradually overexpress amounts of ER (data only shown with SP1 as a representative case, see Fig. 2F, top), confirming the specificity of these antibodies by immunofluorescence. Duplicate control cases also showed strong reproducibility core-to-core.

We also sought to test the MC20 antibody as it is commonly cited in published descriptions of cytoplasmic ER (41–48). We found that, at low exposure, the MC20 antibody appeared to detect a specific 66 kD band on a Western blot analysis of cell line controls (Fig. 2A, top). However, when the blot was left for longer exposure, and compared with SP1, MC20 showed nonspecific immunoreactivity in a series of ER-negative cell lines (Fig. 2A), as well as multiple immunoreactive bands. Similar nonspecific immunoreactivity was observed when MC20 was used to quantify ER by immunofluorescence on the cell line panel (Fig. 2B), in contrast to SP1 (Fig. 2C). On the panel of patient controls present on the same index TMA, QIF analysis of ER expression using MC20 again did not correlate with SP1 (Fig. 2D), and showed strong cytoplasmic staining in cases that were strictly nuclear with SP1 (Fig. 2E). Finally, in a panel of cell lines engineered to overexpress increasing amounts of ER, MC20 showed constitutively high levels of cytoplasmic immunoreactivity, in contrast to increasing nuclear immunoreactivity seen with SP1 (Fig. 2F). To confirm that these data were not because of a poor antibody lot, we repeated each experiment with a second lot of MC20, and found the same results. Thus MC20 was not included in the panel of valid antibodies.

Detection of cytoplasmic ER by multiple antibodies and methods

Once specific and reproducible detection of nuclear ER was confirmed using multiple antibodies, we examined a series of large retrospective cohorts of breast cancer cases on TMAs to determine the frequency of nonnuclear expression of ER. We were able to find cases with strong cytoplasmic immunoreactivity in epithelial cells (colocalized with
cytokeratin, not colocalized with DAPI, Fig. 3A). In all cases, the staining appeared to be more cytoplasmic than membranous, and while a few cases were strictly cytoplasmic without any nuclear reactivity (Fig. 3A, top), in a majority of instances the cytoplasmic staining was observed along with nuclear staining (Fig. 3A, bottom). When subsequent cuts were available (this was the case in our Yale cohorts), we were able to assess expression using multiple ER antibodies and confirm that the cytoplasmic reactivity was observed using more than one antibody (Fig. 3B), suggesting it was
not an epitope-specific artifact, and was potentially full-length ER or an isoform containing an intact N and C terminus. Furthermore, the cytoplasmic immunoreactivity was observed using traditional immunohistochemical methods as well (Fig. 3C), with both N- (1D5) and C-terminal (SP1) antibodies.

Analysis of multiple retrospective cohorts suggests incidence of cytoplasmic ER is low

We have assessed cytoplasmic ER by QIF on nearly 3,200 individual cases from 8 different retrospective breast cancer cohorts, 4 from Yale (YTMA 49, 107, 128, and 130) and 4 from outside sources, including 2 multi-institutional trials (NSABP B14 and South Swedish Breast Cancer Group SBII) as well as 2 others (NCI YTMA 99 and a Vietnamese Oophorectomy cohort). One of these (YTMA 49) was assessed by QIF in duplicate (a second core from each patient) as well as by traditional IHC. In order for a case to be considered "positive" for cytoplasmic ER, 5 conditions had to be met, as described in the Materials and Methods section.

Overall, the incidence of cytoplasmic staining only averaged 1.49%, ranging from 0% to 3.2% at best (Table 1), an incidence that was too low to discover any prognostic or predictive significance, even with relatively large cohorts. As many of the cytoplasmic cases were observed on cohorts from outside institutions, we did not have broad access to the original tissue to conduct any follow-up analysis on the individual cases. We attempted to extract RNA from formalin-fixed, paraffin-embedded (FFPE) cores of the cases found in YTMA 49 and 130 (in Table 1), however, as most of the cases are older than 10 years, the RNA was insufficiently preserved for successful reverse transcriptase PCR analysis with ER-specific primers.

Discussion

We have found that 4 antibodies to ER-alpha (1D5, SP1, F10, and 60c) are highly reproducible and specific by both Western blot as well as QIF analysis, in cell lines and in FFPE cases. Using each of these valid antibodies, we have found
Evidence for cytoplasmic ER in breast cancer specimens, with both immunofluorescence and traditional IHC. However, across nearly 3,200 cases, spanning 8 different retrospective cohorts, we found cytoplasmic ER is present at very low incidence. Thus, its measurement by these methods is unlikely to be of prognostic or predictive value in untreated cases.

Confirmation of immunoreactivity by multiple antibodies suggests that the cytoplasmic localization is not an epitope-specific artifact. However, there are other artifacts that cannot be ruled out, including the possibility that the cytoplasmic localization is a function of fixation method, tissue age, processing, or some other ill-defined preanalytic variable. The specimens assessed in this study came from a variety of institutions and in many cases we have no record of how the tissue was handled. Follow-up studies on many of the cases were not possible as we did not have access to the 6 conditions outlined in the Materials and Methods section. Incidence of cytoplasmic ER was confirmed by 2 separate individuals (A.W. Welsh and D.L. Rimm, one a board-certified pathologist and the other a pathology graduate student) and reported as a percentage of the total cases with valid immunostaining (e.g., cases with missing tissue or out of focus regions were eliminated).

Table 1. Incidence of cytoplasmic ER on multiple retrospective breast cancer cohorts

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Number of cytoplasmic cases</th>
<th>Number of total cases validated</th>
<th>Cases with cytoplasmic staining, %</th>
<th>Antibodies used</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI YTMA 99</td>
<td>17</td>
<td>732</td>
<td>2.3</td>
<td>1D5</td>
</tr>
<tr>
<td>NSABP B14</td>
<td>21</td>
<td>655</td>
<td>3.2</td>
<td>SP1</td>
</tr>
<tr>
<td>South Swedish BCG SBII</td>
<td>0</td>
<td>556</td>
<td>0</td>
<td>1D5</td>
</tr>
<tr>
<td>YTMA 49</td>
<td>2</td>
<td>378</td>
<td>0.5</td>
<td>1D5, SP1, 60c, F10</td>
</tr>
<tr>
<td>YTMA 130</td>
<td>4</td>
<td>316</td>
<td>1.3</td>
<td>1D5, SP1</td>
</tr>
<tr>
<td>YTMA 128</td>
<td>0</td>
<td>183</td>
<td>0</td>
<td>1D5, SP1</td>
</tr>
<tr>
<td>VIE OO cohort</td>
<td>0</td>
<td>156</td>
<td>0</td>
<td>SP1</td>
</tr>
<tr>
<td>TMA 107</td>
<td>3</td>
<td>179</td>
<td>1.7</td>
<td>1D5</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>3,155</td>
<td>1.49</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Eight retrospective cohorts were assessed for the presence of cytoplasmic ER, with multiple validated antibodies, according to the 6 conditions outlined in the Materials and Methods section. Incidence of cytoplasmic ER was confirmed by 2 separate individuals (A.W. Welsh and D.L. Rimm, one a board-certified pathologist and the other a pathology graduate student) and reported as a percentage of the total cases with valid immunostaining (e.g., cases with missing tissue or out of focus regions were eliminated). Abbreviations: BCG, Breast Cancer Group; NCI, National Cancer Institute; NSABP, National Surgical Adjuvant Breast and Bowel Project; OO, oophorectomy; VIE, Vietnamese; YTMA, Yale Tissue Microarray.

In addition, essentially all of the evidence for cytoplasmic ER is shown in preclinical models, most of them cell lines where rapid localization of the receptor was observed. A patient tissue sample, obtained and fixed at a single moment in time, may not be able to detect such a short-lived event. In addition, the majority of cell lines where cytoplasmic ER was observed in a more permanent nature were treated with tamoxifen. It is difficult to test patient tissue after treatment with tamoxifen, as most cohorts are made from primary breast tumors prior to any sort of treatment. In the future, neoadjuvant endocrine therapy cohorts may be able to resolve this issue.

Another explanation for our observations could be the use of TMAs instead of whole sections. If localization of ER is heterogeneous within tumors, it may be seen in whole sections, but missed in low sampling (one-fold redundant) TMA cohorts. While whole sections were not available for any of these cohorts, the use of TMAs also allowed us to maximize the number of specimens included in this study. Furthermore, in smaller series where we assessed ER on whole sections, cytoplasmic ER was not observed (49). We did, however, have triplicate cores for each case in the NCI YTMA 99 cohort, and in cases with evidence for cytoplasmic ER, we were able to observe it in multiple cores. In addition, the largest Yale cohort (YTMA 49) has been produced at 12-fold redundancy. We found that the 2 cases initially positive for cytoplasmic ER were also positive in other cores from the same patient. Furthermore, examination of other cores did not reveal any new cytoplasmic cases when examining other blocks. Finally, it is possible that low levels of cytoplasmic ER exist in many clinical specimens, but the levels of expression are below the threshold of detection using the QIF or immunohistochemical assays. We have not yet determined a way to isolate and quantify ER that is present at very low levels in human tissue specimens.
Our initial goal was to develop an assay that measured cytoplasmic ER to predict patients who would show less benefit from endocrine therapies. However, after extensive antibody validation and examination of a large number of cases, we cannot find evidence for cytoplasmic ER on more than 4% of cases. Although these cohorts are reasonably large, the low event frequency results in insufficient statistical power to look for meaningful associations with outcome.

Disclosure of Potential Conflicts of Interest

D. L. Rimm is a consultant/advisory board member for and holds stock in HistoRx. No potential conflicts of interest were disclosed by other authors.

References


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Allison W. Welsh, Donald R. Lannin, Gregory S. Young, et al.


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