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Comprehensive assessment of estrogen receptor beta antibodies in cancer cell line models and tissue reveals critical limitations in reagent specificity

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ABSTRACT

Estrogen Receptor-β (ERβ) has been implicated in many cancers. In prostate and breast cancer its function is controversial, but genetic studies implicate a role in cancer progression. Much of the confusion around ERβ stems from antibodies that are inadequately validated, yet have become standard tools for deciphering its role. Using an ERβ-inducible cell system we assessed commonly utilized ERβ antibodies and show that one of the most commonly used antibodies, NCL-ER-BETA, is non-specific for ERβ. Other antibodies have limited ERβ specificity or are only specific in one experimental modality. ERβ is commonly studied in MCF-7 (breast) and LNCaP (prostate) cancer cell lines, but we found no ERβ expression in either, using validated antibodies and independent mass spectrometry-based approaches. Our findings question conclusions made about ERβ using the NCL-ER-BETA antibody, or LNCaP and MCF-7 cell lines. We describe robust reagents, which detect ERβ across multiple experimental approaches and in clinical samples.

1. Introduction

Estrogen receptor beta (ERβ) was first discovered in the rat prostate (Kuiper et al., 1996). Since then, there has been considerable interest in understanding its role in both breast and prostate cancer. Despite a large body of literature, the function of ERβ in these two cancers remains unclear (Haldosen et al., 2014; Nelson et al., 2014). Most authors agree that ERβ has a predominantly antiproliferative, pro-apoptotic and tumor-suppressive role (Attia and Ederveen, 2012; Bottner et al., 2014; Chang and Prins, 1999; Ellem and Risbridger, 2007; Horvath et al., 2001; Madak-Erdogan et al., 2013; McPherson et al., 2010; Muthusamy et al., 2011; Nakajima et al., 2011; Rizza et al., 2014; Ruddy et al., 2014; Zhu et al., 2004), however ERβ has also been implicated as an oncogene. This is particularly in the context of Castrate Resistant Prostate Cancer (CRPC) where it has been proposed as a driver of androgen receptor (AR)-dependent gene transcription (Yang et al., 2012, 2015), along with a potential role in mediating the transition from hormone-sensitive to CRPC (Zellweger et al., 2013). In breast cancer, it has been suggested that ERβ may have a 'bi-faceted role' and should not simply be considered a tumor-suppressor (Jonsson et al., 2014). ERβ has been reported to 'cross-talk' with androgen receptor-positive breast cancer (Rizza et al., 2014) and may be an important factor in ERα-negative breast cancer (Gruvberger-Saal et al., 2007; Smart et al., 2013).

Inconsistencies in the reported expression of ERβ in breast and

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prostate cancers as determined by immunohistochemistry (IHC) have contributed to this uncertainty. In prostate, most data support the conclusion that ERβ is highly expressed in benign epithelial cells, with expression declining in cancer development and inversely correlating with increasing Gleason grade (Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Dey et al., 2014; Horvath et al., 2001; Leav et al., 2001; Risbridger et al., 2007). However, it has also been reported that ERβ expression is high in bone and lymph node metastases (Bouchal et al., 2011; Zhu et al., 2004) and that high ERβ expression correlates with poor clinical prognosis (Horvath et al., 2001; Zellweger et al., 2013). In breast cancer, high ERβ expression has been described both as a poor (Guo et al., 2014) and favorable (Esslimani-Sahla et al., 2004; Gruvberger-Saal et al., 2007; Hieken et al., 2015; Leygue and Murphy, 2013; Myers et al., 2004; Omoto et al., 2002; Roger et al., 2001) prognostic marker, with others finding no association between clinico-pathological parameters and ERβ expression (Umekita et al., 2006).

It is recognized that there is wide variability in the sensitivity and specificity of ERβ antibodies, which may contribute to the uncertainties surrounding its molecular action and tissue expression (Choi et al., 2001; Hartman et al., 2012; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012). Previous ERβ antibody validation studies have been published (Carder et al., 2005; Choi et al., 2001; Skliris et al., 2002; Weitsman et al., 2006; Wu et al., 2012), however some of them are limited by reliance on two key assumptions. Firstly, that when assessing an antibody by Western blotting in a cell line model, the factor of interest is expressed and secondly, when assessing an antibody's specificity by IHC in tissue, the tissue expression of the factor has been well characterized. In the case of ERβ, these assumptions are problematic, as its expression in commonly used cell line models and in tissues is not universally accepted (Al-Bader et al., 2011; Asgari and Morakabati, 2011; Attia and Ederveen, 2012; Bouchal et al., 2011; Dey et al., 2014; Gruvberger-Saal et al., 2007; Guo et al., 2014; Hieken et al., 2015; Holbeck et al., 2010; Horvath et al., 2001; Leav et al., 2001; Nakajima et al., 2011; Omoto et al., 2002; Risbridger et al., 2007; Shaaban et al., 2003; Skliris et al., 2002; Umekita et al., 2006; Zellweger et al., 2013; Zhou et al., 2012; Zhu et al., 2004).

In light of this, we sought to test and validate six commonly used, commercially available ERβ antibodies and two non-commercially available ERβ antibodies (Choi et al., 2001; Wu et al., 2012) in a systematic manner that addresses these assumptions. To achieve this, we employed a number of assays for antibody validation, including a novel proteomic-based pull down method called Rapid Immunopreciptation Mass spectrometry of Endogenous protein (RIME) (Mohammed et al., 2013). We then applied successfully validated antibodies to cell line models of breast and prostate cancer commonly used for studies of ERβ to assess them for ERβ expression. ERβ expression in the cell lines was validated by a non-antibody dependent, targeted proteomics method known as Parallel Reaction Monitoring (PRM) (Gallien et al., 2012). Finally, benign and malignant prostate and breast tissues were stained with the validated ERβ antibody to assess tissue expression of ERβ by IHC.

2. Materials and methods

2.1. Cell culture

The cancer cell line MDA-MB-231 with doxycycline-inducible ERβ expression (MDA-MB-231-ERβ) (Reese et al., 2014) was cultured in Dulbeccos Modified Eagle Medium with F12 supplement (DMEM/F12) with 10% heat-inactivated tetracycline-free fetal bovine serum (FBS) (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml blasticidin S (Invivogen) to select for the tetracycline repressor and 500 μg/ml zeocin (Invitrogen) to select for the ERβ expression vector. To induce ERβ expression in MDA-MB 231-ERβ cells, 15 cm^2 plates were seeded with 5×10^6 cells and doxycycline added at either 0.1 μg/ml (for Western blot, real-time polymerase chain reaction (qRT-PCR) and PRM) or 0.5 μg/ml (for RIME) for 24 h. The MCF-7 breast cancer cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated FBS (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. The LNCaP prostate cancer cell line was cultured in RPMI 1640 with 10% heat-inactivated FBS (Fisher-Scientific), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. All cells were incubated at 37 °C with 5% CO2 and cultured to 80–90% confluence. LNCaP and MCF-7 cell lines were obtained from ATCC (Middlesex, UK) and validated by STR genotyping.

2.2. Preparation of mRNA and qRT-PCR

MDA-MB-231-ERβ+, MDA-MB-231-ERβ−, MCF-7 and LNCaP cells were harvested for collection of mRNA using the RNEasy Mini Kit (Qiagen, California USA). On-column DNase digestion was performed to remove contaminating genomic DNA. RNA was quantified with the NanoDrop 8000 (Thermo Scientific, Delaware USA). Samples containing 250 ng random primers, 1 μg RNA, 1 μl 10 mM dNTP mix and water to a total volume of 13 μl were heated to 65 °C for 5 min, followed by 1 min incubation on ice. To each sample 4 μl 5X First-strand buffer, 1 μl 0.1 M DTT, 1 μl RNaseOUT and 1 μl SuperScript III reverse transcriptase (RT) (Thermofisher Scientific, Leicestershire, UK) were added and incubated at 25 °C for 5 min then 50 °C for 60 min followed by heating at 70 °C for 15 min qRT-PCR primers for wild type ERβ (Table 1) were designed based on published sequence of *ESR2* (available from USCS genome browser at http://genome.ucsc.edu/) using the Primer3 software package (Koressaar and Remm, 2007; Untergasser et al., 2012) available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/. UBC primers (SY121212648) were obtained from Sigma-Aldrich (Dorset, UK). Each qRT-PCR reaction contained 7.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems, California USA), 0.5 μl of 10 μM primer mix, 2 μl of a 1:5 dilution of cDNA and nuclease-free water to a final volume of 15 μl. Reactions were performed with the Stratagene Mx3005P RealTime machine in triplicate. Hot-start Taq polymerase was heat-activated at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Fluorescence was read in each cycle and a melting curve constructed as the temperature was increased from 65 °C to 95 °C with continuous fluorescence readings. UBC was used as a control gene to normalize between the samples and relative expression determined using the delta-delta Ct method (Livak and Schmittgen, 2001).

2.3. Western blotting

MDA-MB-231-ERβ+, MDA-MB-231-ERβ−, MCF-7 and LNCaP cells were harvested for nuclear extract using the Ne-Per nuclear extraction kit (Thermo Scientific Pierce, Rockford IL USA) according to the manufacturer's instructions. Extracted protein was quantified using the Direct Detect system (Merrick Millipore, Massachusetts USA). Nuclear extracts were prepared with 4X protein sample loading buffer (LI-COR Biosciences, USA), 10X NuPage sample reducing agent (Thermofisher Scientific, Leicestershire, UK) and water, and 15 μg protein per lane loaded into Bolt 4–12% Bis-Tris gels (Thermofisher Scientific, Leicestershire, UK). Gels were run with MOPS running buffer for 30 min at 60 V followed by 30 min at 120 V. Western transfer was performed using the iBlot system (Invitrogen, Paisley, UK) according to the manufacturer's