Brief Definitive Report

Isoform specificity of progesterone receptor antibodies

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Abstract

Progesterone receptors (PR) are prognostic and predictive biomarkers in hormone-dependent cancers. Two main PR isoforms have been described, PRB and PRA, that differ only in that PRB has 164 extra N-terminal amino acids. It has been reported that several antibodies empirically exclusively recognize PRA in formalin-fixed paraffin-embedded (FFPE) tissues. To confirm these findings, we used human breast cancer xenograft models, T47D-YA and -YB cells expressing PRA or PRB, respectively, MDA-MB-231 cells modified to synthesize PRB, and MDA-MB-231/iPRAB cells which can bi-inducibly express either PRA or PRB. Cells were injected into immunocompromised mice to generate tumours exclusively expressing PRA or PRB. PR isoform expression was verified using immunoblots. FFPE samples from the same tumours were studied by immunohistochemistry using H-190, clone 636, clone 16, and Ab-6 anti-PR antibodies, the latter exclusively recognizing PRB. Except for Ab-6, all antibodies displayed a similar staining pattern. Our results indicate that clones 16, 636, and the H-190 antibody recognize both PR isoforms. They point to the need for more stringency in evaluating the true specificity of purported PRA-specific antibodies as the PRA/PRB ratio may have prognostic and predictive value in breast cancer.

Keywords: progesterone receptor isoforms; breast cancer models; xenografts; specific antibodies; immunohistochemistry

Introduction

There is increasing interest in better understanding a possible differential role for progesterone receptor (PR) isoforms A (PRA) and B (PRB) [1–3], that only differ in that PRB has 164 extra amino acids in the NH2-terminal domain (reviewed in [1,2]). Antibodies exclusively recognizing the unique sequence in PRB are available. Specific antibodies against the PRA isoform, however, are less feasible to design.

In 2001, it was reported that certain anti-PR antibodies, although able to recognize both PR isoforms in immunoblots (western blots), were empirically only reactive with PRA in immunohistochemistry (IHC) [4]. This concept has been used by many laboratories to distinguish the expression of mouse or human PRA isoforms [5–8].

Considering that the PRA/PRB ratio might be important to predict hormone responsiveness [3], and that the western blot assay is not a standard method available in many hospitals to determine this ratio, we decided to evaluate the immunoreactivity of the different commercial anti-PRA or -PRB antibodies suitable for IHC, using different xenograft models engineered to express uniquely either PRA or PRB.

Materials and methods

Animals

NOD/LtSz-scid/IL-2Rgamma null (NSG) mice, 2 months old, originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at IBYME were used. Animal care and manipulation
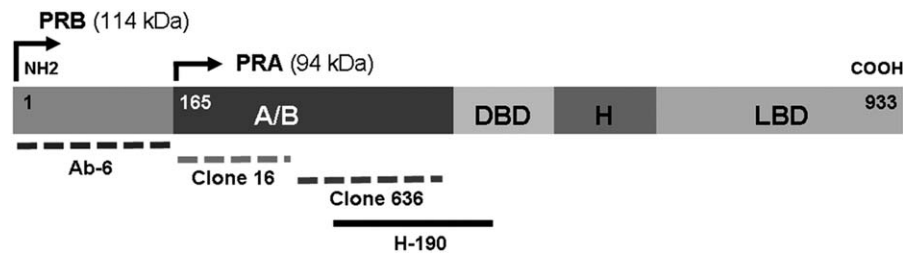
Figure 1. Scheme representing both PR isoforms and the epitope map of antibodies used in IHC studies. DBD: DNA binding domain, H: Hinge, LBD: Ligand binding domain. Dotted lines represent predicted regions; the solid line represents a known epitope. Adapted from Samalecos and Gellersen [13].

were in agreement with the NIH Guide for the Care and Use of Laboratory Animals and by Institutional Guidelines.

Cells

T47D-YA and T47D-YB xenografts were originated as previously described [9]. T47D and MDA-MB-231 cells were purchased from ATCC (Manassas, VA, USA). MDA-MB-231 were transfected with human pSG5-PRB or the empty vector (pSG5), together with a plasmid encoding the neomycin resistance gene (pIRES-N1) [10]. The cells were subsequently cultured with 400 μg/ml G418 (Invitrogen Life Technologies, Carlsbad, CA, USA) and cloned to generate stably transfected cells. MDA-MB-231-iPRAB cells were obtained as described previously [11]. To generate tumours expressing PRB or PRA, tumour cells (5×10^6) were inoculated subcutaneously (sc) into NSG mice. Silastic pellets containing 0.5 mg 17-β-Estradiol (E2) were implanted subcutaneously to generate T47D-YA or -YB xenografts. In the MDA-MB-231-iPRAB model, mice with tumours of 5 mm in the long axis were treated with either doxycycline (DOX; 3 mg/ml and sucrose 1% wt/vol in drinking water) to induce PRB expression, 0.96 mg RSL1 (Exclusive Chemistry Ltd, Obninsk, Russia) intraperitoneally in 0.1 ml of sesame oil to induce PRA expression. After 96 h of treatment, the tumours were excised for PR expression studies. In *in vitro* assays, PRA or PRB was induced as described previously [11] and cells treated for 24 h with medroxyprogesterone acetate (MPA) or Mifepristone (MFP) to evaluate receptor activation.

Western blotting

Extracts were processed as described previously [10]. The cells were lysed using Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The western blotting (WB) membranes were incubated with antibodies against PR (H-190, Santa Cruz Biotech, Dallas, TX, USA), or ERK (sc-94, Santa Cruz Biotech, Dallas, TX, USA) overnight at 4°C.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections were processed [12] and immunostained with PR antibodies: clone 16 (Leica, Buffalo Grove, IL, USA), clone 636 (Dako; Carpinteria, CA, USA), H-190 (Santa Cruz Biotech) or Ab-6 (Thermo Fisher; Waltham, MA, USA) using avidin–biotin–peroxidase complex as described previously [12]. The regions recognized by each antibody are shown in Figure 1, adapted from [13]. T47D-YA and -YB cores and breast cancer cores from a tissue microarray (TMA) [3] were also included. The average number of positive nuclei with respect to total tumour cells in 10 different representative fields of different tumours was quantified (mean ± SD).

Results

Samples of the same tumours were used for WB and IHC studies. As shown in Figure 2A,B, although T47D-YA or -YB tumours express almost exclusively PRA or PRB, respectively, all tumours tested with clone 636, H-190, or clone 16 were positive. Ab-6, as expected, stained almost exclusively T47D-YB tumours. To further rule out that the faint band visible at the PRA molecular weight in the T47D-YB WB could account for the positive staining, we immunostained, using clone 16 and IHC, a TMA composed of breast cancer samples with different PRA/PRB ratios that included cores of T47D-YA and -YB xenografts as controls. The exclusive expression of PRA or PRB of these control xenografts included in the TMA has been shown by WB in a previous study [8]. Intense nuclear staining was observed in both cases and no differences between T47D-YA
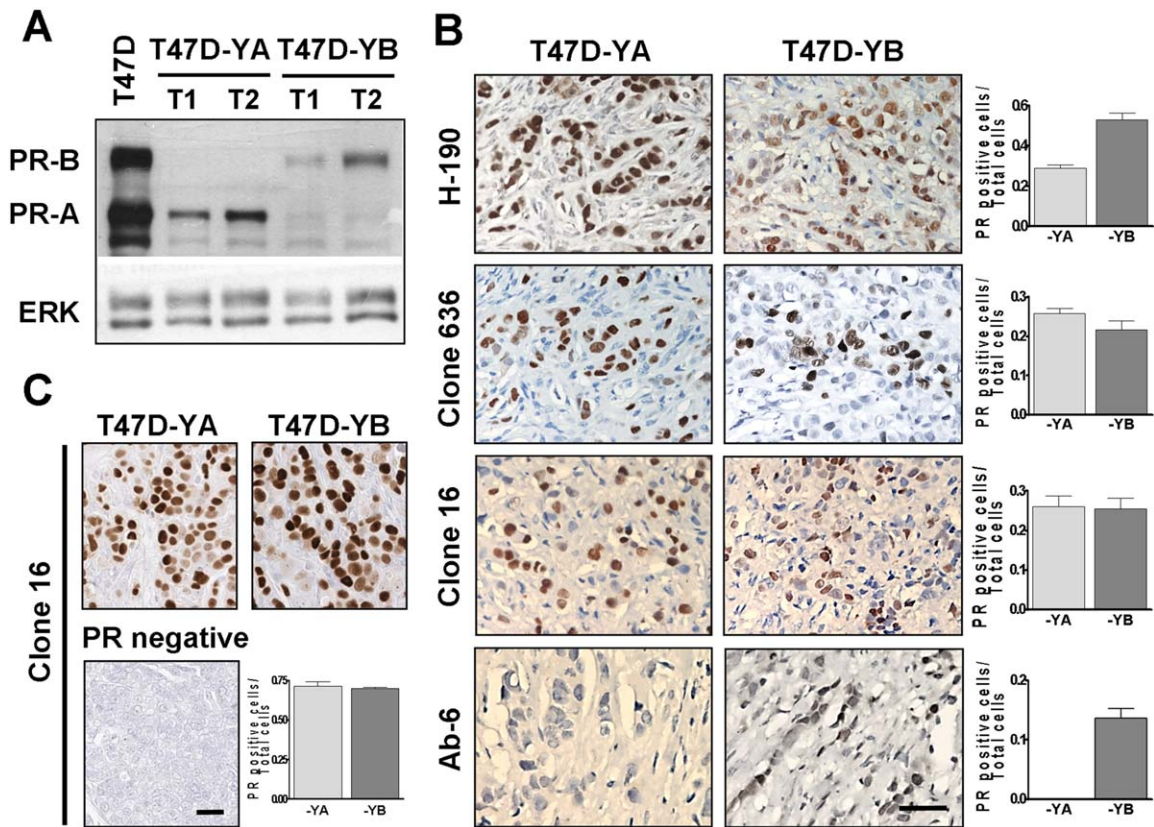
Figure 2. PR expression in T47D-YA (expressing only PRA) and T47D-YB (expressing only PRB) xenografts. (A) Western blot using H-190 antibody (Santa Cruz). T47D tumours that overexpress PRA and PRB were used as positive controls. ERK was used as a loading control. Two different tumours of each type are shown: tumour 1 (T1), and tumour 2 (T2). (B) IHC with four different PR antibodies: clone 636 (Dako), clone 16 (Leica), H-190 (Santa Cruz), and Ab-6 (Thermo-Fisher). All samples showed nuclear staining with all antibodies, except Ab-6 which only stained T47D-YB. Bar: 25 μm. The average number of positive nuclei with respect to total tumour cells in 10 different representative fields of T1 and T2 is shown in the adjacent bar charts ($\pm$ SD). (C) IHC of T47D-YA and –YB xenograft cores from a different experiment than those shown in Figure 2A,B, and a PR negative breast cancer core, the three in the same TMA [3], is shown to reinforce the results shown in Figure 2A,B. The stained nuclei have been quantified as described in B. The western blot of these xenografts was previously shown in Wargon *et al* [8]. Bar: 25 μm.

or -YB xenografts were observed using clone 16 (Figure 2C). These images were included to further emphasize the positive PR staining observed in the -YB xenografts, very similar to that observed in the -YA tumours using the clone 16 antibody. Both cores are in the same TMA, so they were exposed to the same experimental procedures. This intense staining is not due to spurious PRA protein expressed in the YB-tumours.

Similar results were obtained with MDA-MB-231 xenografts engineered either to express only PRB, or with the inducible PRA or PRB cells [11]. Figure 3A shows WB of empty vector- or PRB-transfected MDA-MB-231 tumours. All antibodies gave positive staining with IHC in xenografts expressing only PRB (Figure 3B–D). In control MDA-MB-231 slides, H-190 antibody reacted with cells in the mouse mammary glands (arrow), whereas clone 636 was

only reactive in human tissues (arrows, Figure 3C; left panel).

Figure 4A shows WB of MDA-MB-231-iPRAB cells induced *in vitro* to express PRA or PRB, respectively. A band shift was observed after MPA or MFP treatment, showing that both the agonist and the antagonist are able to induce the typical band shift due to receptor phosphorylation, reinforcing their functionality [14]. When injected *in vivo*, RSL1 or DOX administration-induced exclusive PR isoform expression. Figure 4A right illustrates the WB of the samples used in the IHC assay (Figure 4B). Similar nuclear positive PR staining for H-190, clone 636, and clone 16 was observed in both induced tumours. However, Ab-6 only stained MDA-iPRB samples. The staining was not homogeneous suggesting that the inducer is not equally distributed in the tumour.
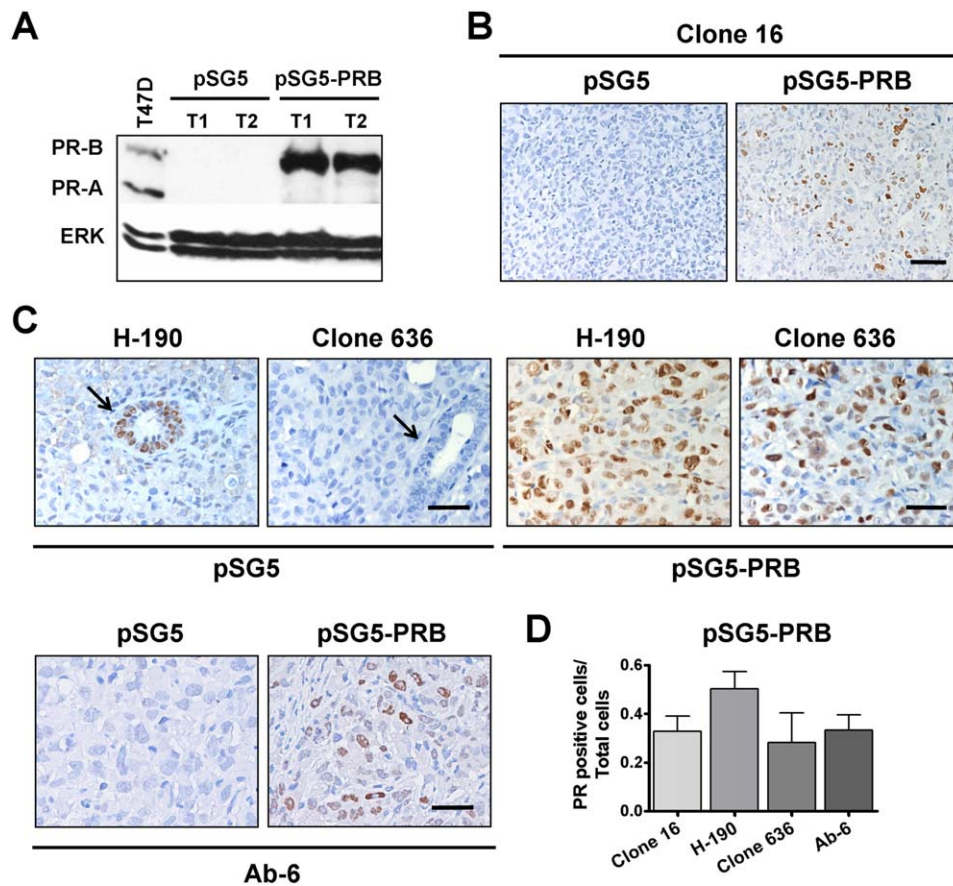
Figure 3. PR expression in MDA-MB-231 xenografts transfected with PRB. (A) Western Blot of two different tumours per group (T1 and T2) using the H-190 antibody. T47D cells were used as a positive control and ERK as a loading control. (B) IHC using clone 16 antibody in MDA-MB-231 xenografts. Bar: 50 μm. (C) PR IHC with H-190, clone 636 or Ab-6 antibodies. H-190 shows nuclear PR staining in the mouse mammary gland; clone 636 is negative (arrows) Bar: 25 μm. The four antibodies (clone 16, clone 636, H-190 and Ab-6) reacted exclusively with PRB-transfected xenografts. (D) The average number of positive nuclei with respect to total tumour cells in 10 different representative fields of T1 and T2 is shown ($\pm$ SD).

In summary, only Ab-6 proved to be PRB specific in IHC studies. Clone 16 antibody is an excellent total anti-PR antibody for IHC, but does not discriminate PRA from PRB.

Discussion

The evaluation of PR expression by IHC has become routine for breast and endometrial cancer management since it is important as an intrinsic prognostic factor. Most commercially available antibodies recognize both PR isoforms except for those, such as Ab-6 or Let 126, generated against the N-terminal region of the PR protein unique to PRB [15,16]. Mote *et al* reported that several antibodies, such as clone 16, or Ab-7, were unable to stain PRB in IHC assays. They

used COS cells transfected with PRA or PRB, MCF-7MII transfected with PRA or MDA-MB-231 with PRB, that were fixed in formalin and embedded in paraffin wax. They suggested that differences in protein folding might be responsible for masking the PRB epitopes [4].

The data shown in the present study demonstrate that these antibodies are not PRA-specific. The plasmids used to generate PRB isoforms in the models used herein were those generated by Kastner *et al* (hPR1), and although they do not have a mutated PRA initiation codon, no PRA isoforms were reported when this plasmid was transfected into different cell lines [17]. If low amounts of PRA were present in our PRB xenografts, and clone 16 reacted with these PR isoforms, the pattern of PR staining would have been much lower than that observed. These assays were performed by two independent
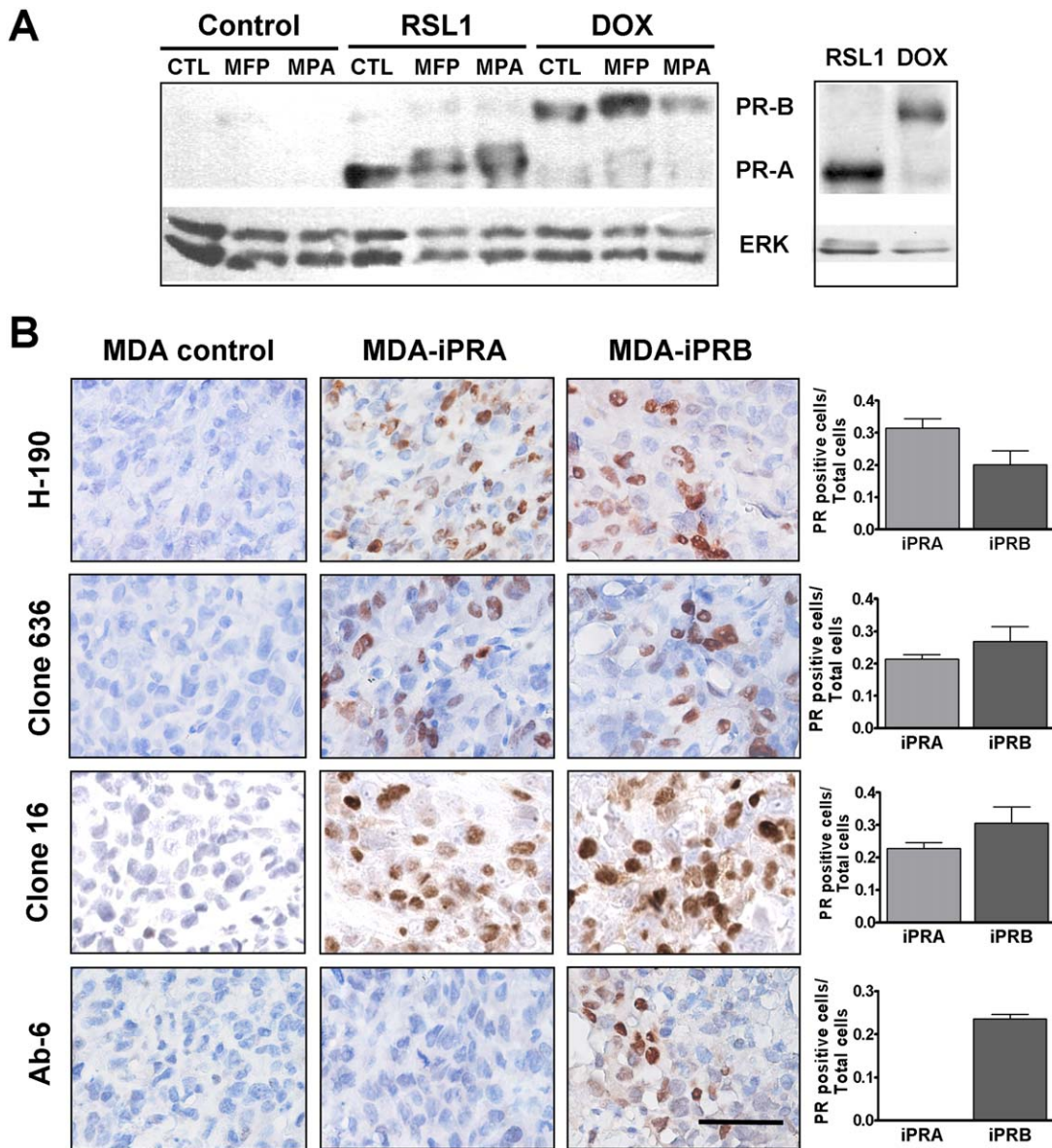
Figure 4. PR expression in samples from MDA-MB-231-iPRAB xenografts expressing PRA or PRB. (A) Left, western blot of MDA-MB-231-iPRAB control cells or cells treated with RSL1 to induce PRA or with DOX to express PRB using H-190 antibody. In addition, cells were treated with MPA (10 nM) or MFP (10 nM) to evaluate functionality of PR (upshifted bands). Right, Representative western blot of a tumour growing in a mouse treated for 96 h with RSL1 to express PRA or with DOX to express PRB. (B) IHC using H-190, clone 636, clone 16, and Ab-6 antibodies. PR staining was observed in areas of both PRA- and PRB-induced tumours, except for Ab-6 that only stained dox-treated tumours. Bar: 30 μm. The average number of positive nuclei with respect to total tumour cells in 10 different representative fields is shown ($\pm$ SD).

laboratories (UCSD and LCH) with similar results. Moreover, we have previously shown strong PR positive staining in formalin-fixed MDA-MB-231 PRB cells in culture by immunofluorescence using Ab-6 and Ab-7 [10].

Since the study of Mote *et al* was published, several others have reported PRA expression in different tissues using the clone 16 or Ab-7 antibodies

[6,7,18–20]; whereas others still preferred to evaluate total PR and PRB expression.

The measurement of PR isoforms is still cumbersome. Due to entangled PRA and PRB regulation including promoter and sequence overlap, measurement of their expression using PCR has also been proposed, yet in many cases the primers used proved not to be appropriate for isoform discrimination or

for quantitative analysis (reviewed in [21]). PRA is measured by substracting the values obtained using primers that quantify total PR (PRA plus PRB) mRNA and those used to quantify PRB mRNA. WB is still the most appropriate method to discriminate PR immunoreactive bands with different molecular weights, with the advantage of using the same antibody that recognizes both PR isoforms with similar affinities in the same electrophoresis gel. However, the possibility of exon deleted variants of PRB that overlap with PRA needs to be excluded.

PR has emerged as a possible therapeutic target in breast cancer, and clinical trials using progestins [22] or antiprogestins (NCT01800422, NCT02651844) are currently underway. We have recently suggested that only those patients with breast cancers expressing levels of PRA higher than those of PRB are amenable to antiprogestin treatment. Determination of the PRA/PRB ratio, as mentioned previously, may have an intrinsic prognostic and predictive value for breast cancer treatment [3]. Thus, the use of validated tools to measure this ratio is mandatory. We consider that those studies reporting the PRA/PRB ratio by IHC or immunofluorescence techniques using these antibodies should be revised since PRA antibodies may also recognize PRB and this may lead to biased conclusions.

Acknowledgements

Author contributions statement

VF, GRS: prepared the T47D-YA/-YB xenografts and VF performed the IHC assays together with MM; MFA, SG: conducted the MDA-MB-231 experiments; BJ, ML: provided the models and participated in discussion; JH, LT, AM: performed the IHC assays at San Diego and participated in the study design; CL: conceived, designed and wrote the manuscript.

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