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Analytical and clinical performance of progesterone receptor antibodies in breast cancer

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ABSTRACT

Objective: Comparison of analytical and immunohistochemical performance of progesterone receptor (PR) antibodies with correlation to recurrence of invasive breast cancer treated with endocrine therapy.
Methods: The binding-affinity kinetics of PR clones 1E2, 1A6, 16 and 636 were compared using synthetic peptides derived from identified epitopes on a Biacore T200. A cohort of 351 cases (Hormone Receptor (HR) +/HER2−) were stained for PR expression with immunohistochemistry (IHC) and scored according to ASCO/CAP criteria.
Results: The stability of the antigen/antibody complex was greater for the 1E2 clone compared to 1A6, 16 and 636 clones. PR IHC on archival tissue resulted in 94.3% (299/317) concordance with clones.
Conclusion: Clones evaluated in this study had a high level of concordance with IHC despite PR (1E2) demonstrating higher analytical binding properties than other clones. In a minority of cases (1.3% for 1E2 and 2.5% for 636) IHC results could convert estrogen receptor (ER)−/PR− to ER−/PR+ tumors, making these patients potentially eligible for endocrine therapy.

1. Introduction

Approximately 75% of breast cancers are ER-positive and 65% are PR-positive [1]. HR IHC is part of the routine evaluation of newly diagnosed invasive-breast carcinoma [2]. Several studies support the importance of PR as a prognostic marker in breast cancer in general [3-7] and specifically, in ER-positive breast cancer [3,8-13]. IHC for ER and PR is also predictive of benefit from endocrine therapy with selective estrogen receptor modulators (SERMs), such as Tamoxifen (TAM), or aromatase inhibitors (AI) in patients with HR-positive tumors [14,15]. The main determinant of eligibility for a SERM or AI is typically ER expression. However, some studies suggest that PR is an independent predictor of outcome in patients treated with endocrine therapy [3,10,16,17]. Conversely, in a large study of AI versus TAM in ER+ breast cancer, PR expression was associated with prognosis, but not with benefit from treatment with an AI [18]. PR expression is often inversely correlated with HER2 expression [19,20] and the absence of PR in ER-positive tumors may be an indicator of aberrant growth factor signaling that may be related to resistance to endocrine therapy [21,22]. However, a large meta-analysis of 20 clinical trials including over 20,000 patients showed that PR was not a useful predictor of response to TAM in ER-positive breast cancer [14].

The current recommendation in the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) guidelines are that all newly diagnosed invasive breast cancers should be tested for both hormone receptors with a cutoff of 1% for positive results for either ER or PR [2]. Prior to the publication of the guidelines in 2010, there were several reports of false negative and false positive IHC results for hormone receptors [23,24].

The majority of PR antibody clones reportedly react with the A and B isoforms of the receptor and routine immunohistochemical studies provide an assessment of total PR protein [25]. The ratio of PRA to PRB (PRA:PRB ratio) is known to be higher in tumor with a worse prognosis [26]. A recent retrospective immunofluorescence IHC study of specimens from the TransATAC trial [27] found, that a high PRA:PRB ratio predicted earlier relapse in women treated with a SERM but not with an

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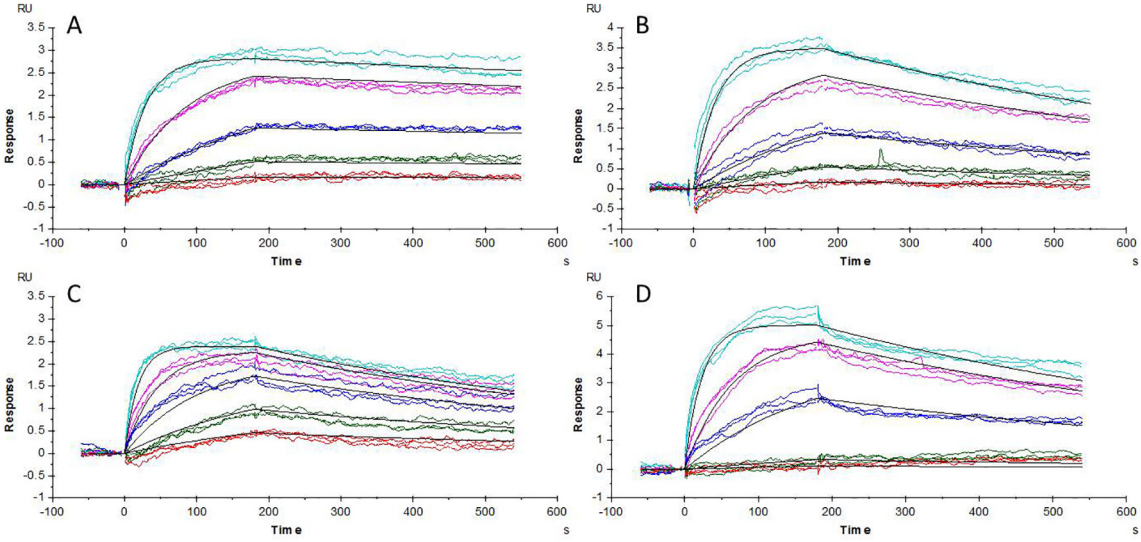

Antibody	Kon (1/Ms)	SE (ka)	Koff (1/s)	SE (kd)	Rmax (RU)	SE (Rmax)	KD (nM)	Half life
Ventana 1E2	2.25E+05	2.10E+02	2.67E-04	1.80E-06	2.8	0.0013	1.2	43
Dako 636	2.05E+05	2.20E+02	1.34E-03	2.30E-06	3.6	0.0018	6.5	9
Leica 1A6	1.40E+05	1.90E+02	1.35E-03	2.70E-06	5.2	0.0029	9.7	9
Leica 16	5.75E+05	8.50E+02	1.51E-03	3.10E-06	2.5	0.0016	2.6	8

Fig. 1. Specific binding curves for the PR antibodies and their associated peptides. The upward slope (K_{on}) indicates the rate at which the antibody binds the peptide. Top of the curve indicates equilibrium. Downward slope is an indicator of dissociation (K_{off}). Each color is a different concentration of peptide. (A) clone 1E2 (B) clone 636 (C) clone 16 (D) clone 1A6. Kinetic coefficients, binding quantities, and standard errors for all PR clones shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AI [28]. PR expression may be an indicator of intact ER-signaling pathways and the absence of PR may be related to aberrant growth factor signaling or promoter methylation [21,22]. Methylation of the PRA promoter has been associated with worse outcomes in patients treated with a SERM. In current practice, laboratories are not called upon to provide isoform-specific data for ER or PR and the role of hormone receptor isoforms in breast cancer remains an area of active investigation. While none of the studied antibodies discern isoform A from B, this suggests the importance of evaluation of PR expression in breast cancers.

Several PR antibody clone comparison studies have shown variability in the performance of commercially available antibody clones [29–31], with recent studies showing concordance between the PR clones despite tumor heterogeneity of PR expression [32,33]. Multiple isoforms of PR have been identified and most commercially available antibody clones recognize PRA and PRB [34] (Supplemental Table 1). The potential for false-negative results in patients who may otherwise benefit from endocrine therapy highlights the need for robust quality assurance schemes to assure inter-laboratory reproducibility for HR IHC testing [35,36].

Since ER/PR status determines the treatment regimen for new breast cancer cases, performance of both antibodies are valuable. Post translational modifications to PR can lead to variation in antibody binding and protein-protein interactions [37]. Binding studies of estrogen have elucidated the mechanisms for receptor formation [38] and alterations due to the presence of ER agonists/antagonists [39,40]. These findings support the need to assess the analytical performance of antibodies intended for clinical use. Analytical characterization techniques, including the measurement of binding kinetics via surface plasmon resonance are well documented in the literature [41–43]. The goal of this study was to compare IHC results for commercially available PR clones and to explore potential differences in sensitivity in a well-characterized cohort of breast cancer cases with outcome data to determine whether clone selection influences PR IHC testing results.

2. Materials and methods

2.1. Epitope mapping of PR clones

Epitope sequences for clones 16, 1E2, 636 and 1A6 were determined through peptide sequence analysis performed by JPT and NimbleGen. In brief, 13–16 amino acid peptide sequences comprising PR isoform B, in single amino acid shifts were queried using the four different clones. Antibodies bound to the peptide array were tagged with species specific, fluorophore conjugated secondary antibodies. In turn these were detected via fluorescent signal. Alignment of the bound peptides was used to determine the overlapping region of bound peptide sequence. The overlapping sequence is representative of the epitope in the peptide.

2.2. Affinity assay

Kinetic analysis was performed on a Biacore T200 using Series S CM5 sensor chips (GE Healthcare, BR100530) with the Amine Coupling kit (GE Healthcare, BR100050). The rabbit antibody capture surface was prepared to a target immobilization level of 15,000 RU with 50 ng/mL goat anti-rabbit IgG Fc (Jackson Labs, 111-005-046) in 10 mM Acetate pH 5. Conditioning was performed with 50 nM whole molecule rabbit IgG (Jackson Labs, 011-000-003) for 120 s at 75 μL/min for five cycles. Surface regeneration was accomplished using a four step

process: 15 s of HBSN (10 mM HEPES and 150 mM NaCl) + 0.5% Tween (Sigma, P9416) at 20 μL/min, 20 s of Glycine pH 1.5 (GE Healthcare, BR100354) at 30 μL/min, and two injections of 30 s each with Glycine pH 1.7 (BioRad, 1610718) at 30 μL/min. The mouse antibody capture surface was prepared to a target immobilization level of 15,000 RU with goat anti-mouse IgG Fc (GE Healthcare, 29215281) according to manufacturer protocols. Conditioning was performed with 50 nM mouse polyclonal (Jackson Labs, 015-000-003) for 120 s at 75 μL/min for five cycles. The capture surface was regenerated according to manufacturer recommendation.

Kinetic measurements were performed at 25 °C in HBSP buffer (10 mM HEPES, 150 mM NaCl, and 0.5% Tween). Antibody capture was done for 120 s at 20 μL/min, and binding curves were generated in triplicate for 180 s association/360 s dissociation at 75 μL/min (see Fig. 1 for clone specific concentrations).

Antibody cross-reactivity was evaluated by flowing 150 nM of each peptide over each non-corresponding antibody at the same capture conditions as mentioned above. Kinetic coefficients were calculated with Biacore T200 Evaluation software using the 1:1 Langmuir binding model with global fit parameters and the bulk refractive index value held constant at 0. Population characteristics were determined according to equations previously suggested by Rich et al. and Gianetti et al. [44,45].

2.3. Case selection and identification

The study was approved by the Cleveland Clinic Institutional Review Board. Study cases included HR + and − by IHC/HER2 FISH negative invasive breast carcinomas. Electronic records dating back more than 10 years were reviewed and 351 cases were identified. The cohort included 90% Stage I–II tumors of which 76% received endocrine therapy and 37% received chemotherapy. 5 μm from formalin-fixed paraffin-embedded (FFPE) sections were analyzed by IHC with PR antibodies. Clinical cases prior to 2008 were initially evaluated for ER and PR expression by IHC using the Labvision SP1 and SP2 clones respectively (formerly Lab Vision/NeoMarkers, Fremont, CA) on a BenchMark Classic (Tucson, AZ) automated platform. These cases, representing approximately 27% of the cohort, were subsequently re-stained using the VENTANA CONFIRM anti-Estrogen Receptor (ER) (SP1) Rabbit Monoclonal Primary Antibody and the CONFIRM anti-Progesterone Receptor (PR) (1E2) Rabbit Monoclonal Primary Antibody in accordance with the package inserts on a BenchMark XT system using *ultraView* Universal DAB Detection Kit (Tucson, AZ). Of the 351 cases, the original HR results were: ER + /PR + 183/351, ER + /PR − 73/351, ER − /PR − 86/351 and ER − /PR + 9/351. Regardless of the results of the re-stain, these patients were analyzed according to their original LabVision clone results, as clinical treatment would have been based on it.

2.4. Clinical sample IHC staining and scoring

5 μm tissue sections were co-mounted on Cardinal Health's Superfrost Plus glass slides (Dublin, OH) along with positive control tissue. Cases were stained with PATHWAY® anti-HER-2/neu (4B5) Rabbit Monoclonal Primary Antibody on a BenchMark XT system in accordance with the package insert using *ultraView* Universal DAB Detection Kit. PR IHC staining was performed with VENTANA CONFIRM anti-Progesterone Receptor (1E2) Rabbit Monoclonal Primary Antibody (Tucson, AZ), DAKO FLEX Anti-Human Progesterone Receptor (636) Mouse Monoclonal Primary Antibody (Dako, Denmark), and the Leica Biosystems Progesterone Receptor (PR) Clone 16 mouse monoclonal antibody (Newcastle, UK). Each of the commercially available PR antibody clones has a different manufacturer, reacts to multiple or single PR isoforms and has a unique epitope position as summarized in Supplemental Table 1. These data represent information provided in the manufacturer's package insert. PR IHC staining with clone 16 was performed using the manufacturer's recommended protocol on the Leica BOND-III staining platform. The CONFIRM anti-Progesterone Receptor (1E2) Rabbit Monoclonal Primary Antibody IHC staining was used in accordance with the package insert on a BenchMark XT system using the *ultra*View Universal DAB Detection Kit. Monoclonal Mouse anti-Human PR Clone 636 (Dako) IHC staining was performed using the Dako Autostainer LINK48 (Copenhagen, Denmark) at Tom Baker Cancer Centre in Calgary, Alberta, Canada according to package insert instructions. All scoring was manually performed by the pathologists according to the interpretation guidelines recommended for each assay in accordance with ASCO/CAP guidelines [2].

3. Results

3.1. Variable epitope mapping of PR antibody clones

Epitope mapping of the PR 1E2, 1A6, 16 and 636 clones identified unique epitopes for each antibody (Supplemental Table 1). Data indicated each clone binds to a different region of the progesterone receptor and the presence of these regions within the various isoforms suggests the individual clones may bind multiple PR isoforms. While the ability to detect the various isoforms is apparent, the detection of these isoforms in breast tumor tissue would be dependent on the individual expression profiles.

3.2. Kinetic analysis and binding affinity

As tested, the peptide concentrations evaluated spanned the dynamic range from near R_{max} to the signal threshold of each clone. Binding 1:1 was observed for all interactions except clone 636, which showed an observable amount of heterogeneous binding not accounted for in the curve fits. The surface plasmon resonance data demonstrated no quality concerns as there were no antibody diffusion anomalies and small standard errors relative to the kinetic values (Fig. 1).

Binding kinetic analysis indicates a 5× greater affinity for the PR 1E2 clone compared to the 636 clone due primarily to the observed affinity dissociation rate. While this suggests a more stable antibody antigen complex, a five-fold increase would not be expected to result in a significant change in the performance of PR 1E2 IHC. It is expected that a 10× greater affinity would be needed to generate a significant change with this antibody. The 5× affinity observed here would suggest minor differences in staining or the ability to potentially detect low levels of the PR proteins. Clone 16 binds the peptide faster than other clones tested, while clone 1E2 has the slowest dissociation rate of the clones tested (Fig. 1). A lack of binding response was observed between the clones and their non-target peptide sequences (Supplemental Fig. 1). Affinity (KD) values with antibody specific peptides were found to be very similar among all four clones for the binding conditions tested, with the greatest difference being eight-fold greater affinity with the 1E2 clone compared to the 1A6 clone (Fig. 1). Clone 16 had the greatest on rate. Clone 1E2 has the slowest off rate; roughly five-fold lower than clone 16. Molar ratio binding calculations for the antibody populations suggest clones 1E2, 636, and 1A6 are achieving half the full avidity binding potential, which is reflected by less than 50% antibody activity. Clone 16 achieves one quarter full binding potential. The decreased off rate of clone 1E2 compared to the other clones translates to an increased relative half-life, which indicates a longer lasting antibody-antigen complex.

3.3. Concordant IHC PR study cases

Two pathologists reviewed all IHC stained cases. In case of disagreement, a third pathologist provided an additional blinded assessment so all cases had two pathologists alignment on positive/negative status. Overall, staining observed between the clones was similar (Fig. 2). Of the 351 cases, 317 had at least two pathologists' scores

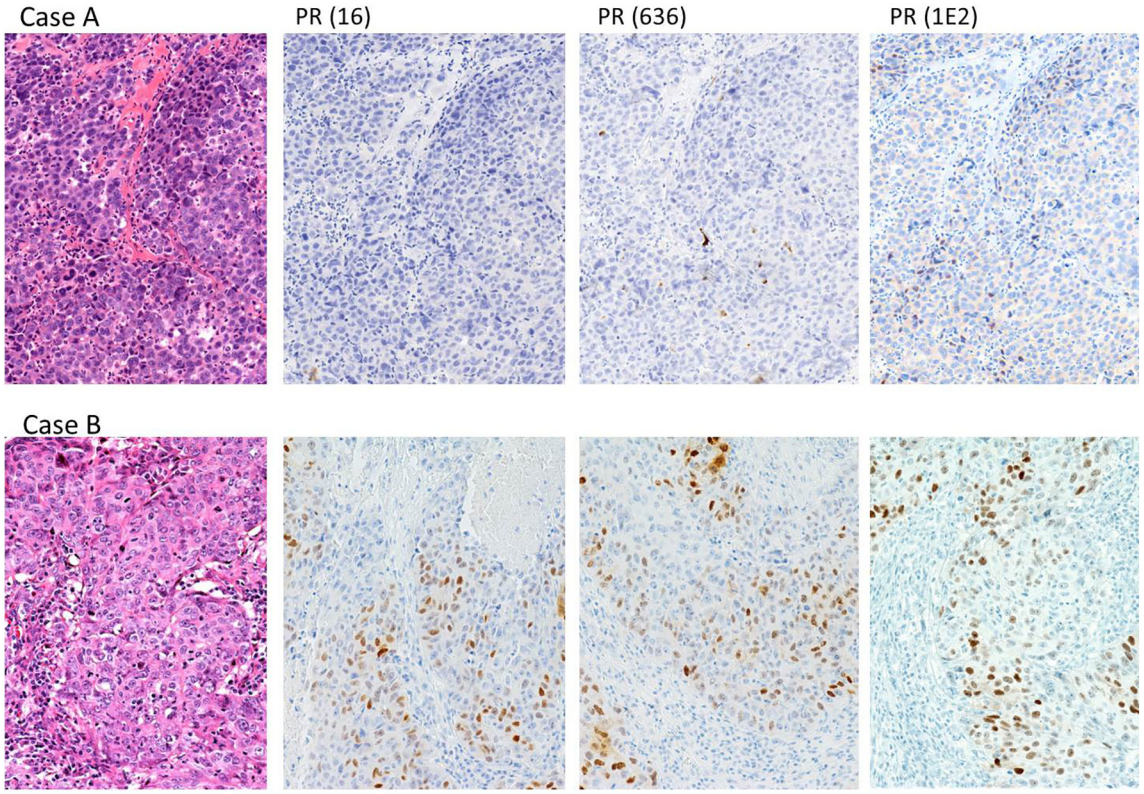

Fig. 2. Representative concordant PR staining of low and high expression cases evaluated in this study with associated H&E stain. 20× magnification.

Table 1
Discordant cases compared across PR clones. Positive discordant is defined as a positive result when both other clones assessed were negative. Negative discordant is defined as a negative result when both other clones were positive. Total cases with a minimum of two pathologist reads available was 317 of the total cohort of 351.

	Ventana 1E2	Dako 636	Leica 16	All clones N = 317
Positive discordant cases (%)	4 (1.3%)	8 (2.5%)	0	12 (3.8%)
Negative discordant cases (%)	2 (0.6%)	0	4 (1.3%)	6 (1.9%)
All discordant cases (%)	6 (1.9%)	8 (2.5%)	4 (1.3%)	18 (5.7%)

available. Pathologist scores based on ASCO/CAP criteria were concordant in 299/317 (94.3%) cases between clones 1E2, 636 and 16 that were either all positive or all negative. Of the 317 cases, there were four (1.3%) cases which were PR positive with the 1E2 clone and negative with both the 636 and 16 clone (Table 1). One of these cases did not get endocrine therapy and had a distant recurrence, the follow up for the other three patients is unknown. There were eight (2.5%) cases which were PR positive with the 636 clone and negative with both the 1E2 and 16 clone, only two did not get hormone therapy and had a distant recurrence, while five cases received hormone therapy and did not have a recurrence, and the follow up for one patient was unknown. There were no cases that were positive with the 16 clone that were negative with both 636 and 1E2. The overall recurrence rate within the cohort was 13.3% (42/317) with 32 distant recurrences, 9 local, and 1 of unknown type.

4. Discussion

A comparison of four PR clones identified four different epitope locations, comparative dissociation rates and comparative binding affinity. The epitope mapping indicated clone 16 could potentially bind to isoforms A and B, while clones 1E2, 636, and 1A6 could bind isoforms A, B and C. The analytical results correlated to the clinical immunohistochemistry results, demonstrating concordant staining outcomes across three clones with only a few cases showing discrepancy.

All four clones exhibited roughly equivalent single nanomolar binding, which is typical for antibodies used in IHC [46]. While these antibodies are usually run at 37 °C on automated platforms, the kinetic comparisons were performed at 25 °C due to a mass transport driven reduction in data quality at higher temperatures. Typically, increases in antibody incubation temperatures are accompanied by concurrent increases in both association and dissociation rates. The values described here can be expected to serve as reasonable relative comparators for the four clones at functional temperatures.

Unique characteristics of the clones were seen in their kinetic binding affinity, with a lower dissociation rate of clone 1E2 as compared to the others. Saturation of epitope sites can be achieved with clones of reasonably lower affinity by increasing antibody titer or incubation time. However, the dissociation rate of an antibody from its target epitope is an indicator of the lasting stability for the resulting complex and the half-life calculations listed in Fig. 1 were derived from observed kinetic dissociation rates. It is the formation and the maintenance of the antibody/antigen complex, at least through the detection step of the assay, that results in the staining observed. Since three of the four antibodies tested demonstrated half-lives of less than 10 min, it could be expected that a certain amount of avidity interactions are occurring in stained tissue. If the peptide sequences used in these kinetic measurements mimic the functional epitope, additional work would be required to evaluate the tissue-specific sensitivity impact of avidity versus affinity interactions within these assays.

The kinetic and dissociation differences observed are only a single component contributing to potential variation in staining patterns seen between these antibodies. In the immunohistochemistry process, there are many other variables to account for. Factors such as pre-analytics and tissue preparation for each case can affect staining outcomes. Other issues include differences in detection chemistry and variations in

automated platforms that can result in staining differences between each assay.

Previously, studies have compared the performance of different PR antibody clones. Press et al. identified that mouse monoclonal antibodies for clone 636 and 1294 exhibit the highest staining intensity and proportion of positive cells in FFPE tissue [32]. PR status as determined by clone 636 was the comparator in the United Kingdom National External Quality Analysis (UK NEQAS) study that identified the potential for false positive results with the rabbit monoclonal antibody SP2 [24]. The study also indicated that the rabbit monoclonal antibody 1E2 achieved an overall higher acceptable pass rate than mouse monoclonal 1A6 clone and rabbit monoclonal SP2 clone as compared to mouse monoclonal antibody 636. Studies comparing mouse and rabbit monoclonal antibodies have reported more categorical positives as well as higher intensity and a higher percentage of cells staining with rabbit monoclonal antibodies [29,30]. A recent analysis of 532 cases in the Calgary Tamoxifen Breast Cancer Cohort showed a trend toward an association with 5-year disease-free survival (DFS) with the rabbit monoclonal antibody 1E2 [47]. These studies strongly support the requirement for laboratories that perform these tests to document their quality assurance data and participate in external proficiency testing programs. CAP recommends and administers the Q-PROBES program to maintain consistent laboratory standards [48].

We also compared the PR IHC positivity for three PR clones (1E2, 636 and 16) relative to prediction of recurrence in a treated cohort of invasive ductal carcinoma patients. Overall there was a very high level of concordance for PR IHC staining (299/317, 94.3%). There were, however, some discrepant cases observed for all clones analyzed. The need for routine analysis of PR in all newly diagnosed breast cancers has been debated in the literature [47,49,50]. In particular, routine clinical testing for PR identifies a subset ER-negative, PR-positive tumors corresponding to 1–4% of cases [16,50–53]. Studies have shown that patients with ER-negative, PR-positive tumors may have adverse clinicopathological factors [52] and a higher risk of recurrence and shorter overall survival as compared to ER-positive, PR-positive and ER-negative, PR-negative tumors [16,51]. Some studies have suggested that this group of patients is likely to be younger, premenopausal [16,53] and less likely to benefit from TAM when compared to ER-positive, PR-positive patients [54]. Other studies indicate that patients with ER-negative, PR-positive, low-grade tumors may benefit from a SERM but the survival benefit may be restricted to low-grade tumors [55]. In contrast, the ER-negative, PR-positive phenotype is currently being debated in the literature, as PR messenger RNA levels have been reported to be very low in ER-negative breast cancers. Use of PR positivity as a determinant for potential response to hormone-targeted therapies has been questioned for ER-negative breast cancers, but there is not sufficient data to determine this [50]. Nevertheless, the ASCO/CAP guidelines recommend testing for ER and PR in all newly diagnosed invasive breast cancers [2]. The data in our series raises the possibility that the rabbit monoclonal antibody 1E2 may be analytically more sensitive for PR, but results in similar clinical results as other clones available. All PR clones assessed had some discordance with other clones, however in general concordance was very high. A positive PR status may convert ER−/PR− tumors to ER−/PR+ resulting in eligibility for endocrine therapy, but the benefit from endocrine therapy in this subset of patients has not been established.

5. Conclusion

Among PR antibodies assessed, Clone 16 had the fastest on rate while the 1E2 clone showed the slowest off rate and highest affinity for PR in binding kinetic analysis. Despite different affinity results, all clones performed similarly in the context of IHC with a minority of cases (1.3% for 1E2 and 2.5% for 636) where PR staining results converted ER−/PR− patients to ER−/PR+, potentially making them eligible for endocrine therapy. Expanding the cohort size and extending the follow-up period will be required to determine the clinical significance of these findings.

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Conflict of interest disclosure

Reagents and other consumables were provided by Ventana Medical Systems, Inc, (A member of the Roche Group). Authors BM, DW, MS, EW, AHN, and RJ are employed by Ventana Medical Systems, Inc.

Prior presentation

Preliminary data were presented at the European Congress of Pathology, September 25–29, 2016 in Cologne, Germany and at the Annual Meeting of the United States and Canadian Academy of Pathology, March 4–10, 2017, in San Antonio, TX.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anndiagpath.2018.02.007.

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