Int J Clin Exp Pathol 2014;7(1):331-339
www.ijcep.com /ISSN:1936-2625/IJCEP1309072

Original Article

Evaluation of the progesterone receptor status in breast cancer using three different antibodies: a comparison by Allred score system

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Received September 25, 2013; Accepted October 15, 2013; Epub December 15, 2013; Published January 1, 2014

Abstract: Breast cancer (BC) hormonal receptors status is assessed by immunohistochemistry (IHC), a specific, sensitive, and accessible method that guide breast cancer treatment. In this study, we evaluated progesterone receptor (PR) expression in 53 BC cases using 3 anti-PgR antibodies (AB): monoclonal (SP42 and PgR636) and polyclonal ab62621. Primary BC cases (with signed informed consent) were used to generate tissue microarray platforms, where PR expression was accessed by IHC and evaluated by the Allred score. Categorical and quantitative data are shown in percentage and mean, respectively. Concordance (CON) and correlation among ABs were analyzed by Kappa factor (K), Spearman's correlation coefficient (ρ) or intraclass correlation coefficient. Staining patterns of each AB were compared by paired T-Test. We noted poor CON and K between ab62621 vs SP42 (CON=64.1%; K=0.247), and ab62621 vs PgR636 (CON=62.3%; K=0.204), but higher CON between SP42 vs PgR636 (CON 90.6%; K=0.738). Data were corroborated by Mc Nemar statistical test (p=0.019, p=0.014 and p>0.05, respectively). Regarding staining intensity (SI) among PgR+ samples, we found higher proportion of weak staining and lower SI for ab62621 (48.3%; mean IS=1.6), when compared to SP42 (20.0%, mean IS=2.1, T-test p<0.01) and PgR636 (2.3, 21.9%, T-test p<0.01). Within the entire sample, similar results were observed following ρ: SP42 vs PgR636 (ρ=0.8103); ab62621 vs SP42 (ρ=0.3524); ab62621 vs PgR636 (ρ=0.4075). As for proportion of stained cells and proportion score (PS), among PgR+ samples, the mean values for ab62621 (75.4%; 4.8) were significantly higher than those of SP42 (56.3%, 4.3; T-test p<0.01) and RPG636 (60.1%; 4.2; T-test p<0.01). Similar data were found after analyzing PS for the entire sample: SP42 vs PgR636 (ρ=0.8588); SP42 vs ab62621 (ρ=0.4832); RPG636 vs ab62621 (ρ=0.4050). Our data indicate that anti-PgR monoclonal ABs, PgR636 and SP42, are, unlike ab62621, equally suitable to test BC PgR status by IHC due to their higher accuracy. Therefore, we suggest their clinical use during BC diagnosis; thus, enabling more precise therapeutic decisions to treat BC.

Keywords: Progesterone receptor, antibodies, breast cancer

Introduction

Breast cancer (BC) therapeutic decision is currently guided by patients' clinical information, histomorphological features of the tumor tissue, and, importantly, by the determination of the expression of hormonal receptors (HR) (estrogen alpha and progesterone receptors; ERα and PR, respectively) and of the oncoprotein HER2 by tumor cells [1]. Whereas the expression of ER by tumor cells confers better prognosis to the patient that can be treated with endocrine therapy, which is based on selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, or aromatase inhibitors as anastrozole and letrozole [2, 3], HER2+ BC cases are eligible for HER2-targeted therapy either with anti-HER2 monoclonal antibodies, as trastuzumab, or tyrosine kinase inhibitors, as lapatinib [4, 5]. Ultimately, then, BC accurate diagnosis is crucial for the prediction of patient's clinical outcome.

PR is a target molecule of ERα signaling pathway in several cell types, including breast malignant epithelial cells; therefore, PR+ BC cases are considered to be ER-dependent, and to have functional ERα pathway. Whereas ER+, PR+, HER2- BCs are considered better prognosis disease [6], PR- or HER2- tumors are more likely to be aggressive [7, 8]. Therefore, the precise evaluation of PR status during BC diagnosis is of clinical interest because it provides an auxiliary tool to direct patients to endocrine therapy regardless of the disease ER status following immunohistochemistry (IHC) analysis [9]. In this context, patients with ER-/PR+ and ER+/PR+ tumors are generally considered as candidates for endocrine therapy; on the other hand, clinically, ER-/PR+ cases seem to benefit less from adjuvant tamoxifen than ER+/PR+ [10].

Despite the specificity, sensitivity and accessibility of the evaluation of BC HR status following IHC assays, different ER and PR antibodies have been correlated to discrepant results [11-13]. This clearly demonstrates the need to systematically compare the results obtained with different ER and PR antibodies, thus providing evidences of the most accurate reagent to be used in BC clinical routine. Our group has recently proved that the evaluation of BC ERα status is more precise with the SP1 than with the 1D5 ER monoclonal antibody [10]. Indeed, we have observed higher intensity scores, therefore higher Allred scores, with SP1 than with 1D5 ER monoclonal antibody, thus decreasing the possibility of ERα status misinterpretation and, consequently, inappropriate BC treatment that would compromise the patients' quality of life and overall survival [14].

Although very useful in clinical routine, there is no conclusive recommendation in the literature about which antibodies would be more specific and sensitive for evaluating PR in BC samples by IHC [15]. The most frequently used antibodies for HR evaluation are mouse monoclonal antibodies. Recently, a generation of rabbit monoclonal antibodies has been developed [16]. The technology to prepare these antibodies from a single hybridoma allows the production of antibodies with high sensitivity and specificity according to the manufacturers. These characteristics allow higher working dilutions and, consequently, a lower cost per test [17, 18].

Altogether, there is a strong body of evidences to support that the precise evaluation of HR status in BC biopsies following IHC assays is imperative in defining the therapeutic strategies to combat the disease, as well as the clinical outcome of patients. Taking into consideration that the antibody used in IHC analysis of BC samples influences the ultimate pathologist's interpretation of the tumor cells HR status, in the present study, we assessed PR expression in 53 BC cases, by IHC, using the anti-PR antibodies Sp42 (rabbit monoclonal), ab62621 (rabbit polyclonal), and PgR636 (mouse monoclonal). The expression of PR within the studied BC population was compared by assessing the percentage of cells stained within the analyzed sample, as well as their staining intensity by applying the Allred score system.

Materials and methods

Samples and cohort definition

Fifty three BC samples were obtained from the two public oncology reference hospitals in the state of Espírito Santo (Brazil), Cassiano Antonio de Moraes Hospital and Santa Rita de Cássia Hospital. All cases were revised by a pathologist, and the BC diagnosis was confirmed. All living patients gave written informed consent before enrollment in the study. This work was approved by the Human Research Ethics Committee of the Federal University of Espirito Santo.

Tissue array platforms and progesterone receptor immunohistochemistry assays

Hematoxylin and eosin stained sections from each breast sample were reviewed by a pathologist to confirm the primary BC diagnosis, and to select one representative 2 mm area of the tumor embedded in a paraffin block for immunohistochemical analysis. Each 2 mm sample was further transferred to a transient tissue array platform, generating our customized BC tissue array platforms [14, 19-21]. The immunohistochemistry assay followed a protocol optimized by our group in 5 μm sample sections of the generated BC tissue array platforms [20-22]. Briefly, after removal of paraffin, sections were immersed in preheated antigen-retrieval solution (Tris/EDTA 1X, pH=9), incubated at 95 °C- 99 °C for 30 minutes, and then allowed to cool down to room temperature for 30 minutes.

Table 1. Primary breast cancer cohort classified according to the tumor histological type

Histologic Type	Nº (Percentage of tumors)
Invasive ductal carcinoma	40 (75.5%)
Invasive lobular carcinoma	4 (7.5%)
Mucinous carcinoma	1 (1.9%)
Microinvasion ductal carcinoma	1 (1.9%)
In situ ductal carcinoma	6 (11.3%)
Medullary carcinoma	1 (1.9%)

Table 2. Stratification of the PR+ breast cancer cases population by the proportion of stained cells, staining intensity and A-score

	Rmab (SP42)	Mmab (636)	Rpab (62621)
	N (%)	N (%)	N (%)
Negative	13 (24.5)	12 (22.6)	24 (45.3)
Positive	40 (75.5)	41 (77.4)	29 (54.7)
Proportion of stained cells			
1-25%	7 (17.5)	6 (14.6)	0 (0.0)
26-50%	10 (25.0)	8 (19.5)	4 (13.7)
51-75%	15 (37.5)	12 (29.3)	7 (24.1)
>75%	8 (20.0)	15 (36.6)	18 (62.1)
Staining Intensity			
Weak	8 (20.0)	9 (21.9)	14 (48.3)
Moderate	18 (45.0)	12 (29.3)	7 (24.1)
Strong	14 (35.0)	20 (48.8)	8 (27.6)
A-Score			
3	3 (7.5)	2 (4.9)	0 (0.0)
4	5 (12.5)	5 (12.2)	1 (3.5)
5	3 (7.5)	3 (7.3)	3 (10.3)
6	6 (15.0)	3 (7.3)	12 (41.4)
7	10 (25.0)	10 (24.4)	11 (37.9)
8	13 (32.5)	18 (43.9)	2 (6.9)

Then, sections were incubated for 3 hours with the primary antibodies of interest: rabbit monoclonal anti-human PgR, clone SP42 (Spring Bioscience, Pleasanton, CA), dilution 1:100, mouse monoclonal anti-human PgR, clone PgR636 (Dako Cytomation, Carpinteria CA) dilution 1:100, or rabbit polyclonal polyclonal anti-human PgR, clone ab62621 (Abcam Cambridge, MA, USA) diluition 1:100. Sections were incubated with biotinylated universal secondary antibody (Dako Cytomation LSAB+ System-HRP, Dako Cytomation, Carpinteria CA) for 30 minutes. Endogenous peroxidase activity was blocked by a 5 minutes incubation of the slides in 3% (v/v) hydrogen peroxide. Antigen-antibody complexes were detected by the avidin-biotin-peroxidase method, using 3,3-diaminobenzidine (DAB) as the chromogenic substrate. The sections were incubated with streptavidin conjugated to peroxidase (LSAB+ System-HRP, Dako Cytomation, Carpinteria CA) for 30 minutes and, then, with DAB (Liquid DAB+ Substrat Chromogen System, Dako Cytomation, Dako Cytomation, Carpinteria CA) for 5 minutes. Slides were counterstained with hematoxylin and immersed in 5% (p/v) ammonium hydroxide. Positive controls were included in the BC tissue array platform in opposite positions of the chip in order to assure technical accuracy of PR expression in breast tissue. Negative control experiments were conducted in the absence of the primary antibodies listed above, in parallel of all assays. Cases with less than 1% nuclear staining in tumor cells were considered negative for the expression of the protein of interest. Subcellular localization was also noted.

The scoring system proposed for the analysis of the 4 tissue array platforms was the Allred score. This method categorizes the samples based on a proportion score (PS) that classifies the cases into 6 groups according to the percentage of stained cells, and on an intensity score (IS) that divides them into 4 classes according to staining intensity. The PS groups are as follows: Score 0: negative; Score 1: <1%; Score 2: 1%-10%; Score 3: 10%-33.3%; Score 4: 33.3%-66.6%; and Score 5: >66.6%. The IS classes are, in turn: Score 0: negative; Score 1: weak; Score 2: intermediate; and Score 3: strong. Finally, the two scores are combined, and the total score (TS) is given, considering an A-Score of 0 or 2-8 [19]. The IHC reactions were interpreted by the same pathologist with notorious expertise in breast pathology.

Statistical analysis

Categorical data are shown in percentage, while quantitative data are shown as mean.

Table 3. Mean proportion of stained cells, proportion score, intensity score, and A-Score for the entire sample, and the two main histological types

	Rmab (SP42)	Mmab (636)	Rpab (62621)	*p*-value		
				a	b	c
All cases (n=53)						
Positive staining	40	41	29			
Mean proportion of stained cells (%)	56.3	60.1	75.4	ns	<0.01	<0.01
Mean proportion score	4.2	4.3	4.8	ns	<0.01	<0.05
Mean intensity score	2.1	2.3	1.6	ns	<0.01	<0.001
Mean A-Score	6.3	6.6	6.3	ns	ns	ns
IDC (n=40)						
Positive staining	31	32	22			
Mean proportion of stained cells (%)	53	58.3	71.3	ns	<0.05	0.077[d]
Mean proportion score	4.1	4.2	4.7	ns	<0.05	0.056[d]
Mean intensity	2.1	2.2	1.5	ns	<0.05	<0.01
Mean A-Score	6.2	6.5	6.2	ns	ns	ns
ISDC (N=6)						
Positive staining	5	5	4			
Mean proportion of stained cells (%)	66.4	66.8	78.8	[e]	[e]	[e]
Mean proportion score	4.6	4.6	5.0	[e]	[e]	[e]
Mean intensity score	2.6	2.6	1.7	[e]	[e]	[e]
Mean A-Score	7.2	7.2	6.8	[e]	[e]	[e]

[a]T-test comparing RmabSP42 and Mmab636. [b]T-test comparing RmabSP42 and Rpab62621. [c]T-test comparing Mmab636 and Rpab62621. [d]Limitrophe *p*-value. [e]No statistical test was applied due to small N.

Concordance and correlation among antibodies were accessed by calculating Kappa factor, Spearman's correlation coefficient or intraclass correlation coefficient. Staining patterns for each antibody were compared by paired T-Test.

Results and discussion

In the present study, fifty three paraffin embedded primary BC biopsies (75.5%, 11.3% and 7.5% of the cases were invasive ductal, in situ ductal and invasive lobular carcinomas, respectively - **Table 1**) were collected, and PR expression was assessed by IHC using three different anti-PR antibodies: a rabbit polyclonal antibody (62621), a rabbit monoclonal antibody (SP42) and a mouse monoclonal antibody (636).

While analyzing the IHC results for PR expression amongst the BC biopsies, we observed lower tendency of positive results when the slides were probed with the anti-PR rabbit polyclonal antibody 62621, when compared with the monoclonal antibodies (both rabbit, SP42, and mouse, 636). In fact, we obtained 54.7% (29/53), 75.5% (40/53) and 77.4% (41/53) of PR+ BC cases following IHC assays using the anti-PR antibodies 62621, SP42 and 636, respectively. As a consequence, there were poor concordance (CON) and kappa factor (κ) between 62621 and SP42 (19 discordant results - CON=64.1%; κ=0.247), as well as between 62621 and 636 (20 discordant results - CON=62.3%; κ=0.204). On the other hand, the two monoclonal antibodies, SP42 and 636, described higher concordance (5 discordant results - CON 90.6%; κ=0.738). Mc Nemar statistical test supported the discordance between 62621 and SP42 (p=0.019), and between 62621 and 636 (p=0.014). Similarly, the test provided no statistically differences among SP42 and 636 (p>0.05). Data is shown in **Table 2**.

Similar results were obtained by Chebil and collaborators [12], in which study the inter-observer reproducibility of IHC assessments of ER and PR was analyzed in more than 120 cases of primary BC. It is important to address that, as a consequence of the authors' work, anti-PR polyclonal antibodies are no longer used in the diagnosis routine of the collaborator laboratories. It is remarkable to inform that the present study has also lead to the exclusive use of anti-

PR monoclonal antibodies in the hospitals we collaborate; therefore, improving the accuracy of BC diagnosis and patients' overall quality of life.

Further stratification of our cohort to evaluate exclusively the PR+ BC cases revealed a higher proportion of weakly stained samples (48.3%) following the use of the rabbit polyclonal antibody 62621 when compared to the two monoclonal antibodies, mouse 636 (21.9%) and rabbit SP42 (20.0%) (**Table 2**). The determination of the mean intensity score (IS) supported the findings. IHC experiments run with the rabbit polyclonal PR antibody 62621 provided mean IS value of 1.6, which is significantly lower than the mean IS values observed with the monoclonal antibodies rabbit SP42 (2.1, T-test $p<0.01$) and mouse 636 (2.3, T-test $p<0.01$) (**Table 3**). There was relatively good correlation between the two monoclonal anti-PR antibodies, rabbit SP42 and mouse 636, as shown by the Spearman's correlation coefficient ρ ($\rho=0.8103$). Nonetheless, there was poor correlation between the rabbit polyclonal anti-PR antibody 2621 and the rabbit monoclonal anti-PR antibody SP42 ($\rho=0.3524$), and between 62621 and the mouse monoclonal anti-PR antibody 636 ($\rho=0.4075$) (**Table 3**).

Still evaluating the PR+ BC cases, but focusing on the mean proportion of stained cells within the biopsy core, a higher number of positive cells was obtained when the slides where probed with the polyclonal anti-PR 62621 antibody (75.4%) than with the monoclonal anti-PR antibodies rabbit SP42 (56.3%, T-test $p<0.01$) and mouse 636 (60.1%, T-test $p<0.01$) (**Table 3**). There was no statistical differences between the two monoclonal antibodies tested (**Table 3**). Accordingly, we observed that 62.1% of the PR+ BC samples had more than 75% of stained cells when the rabbit polyclonal antibody 62621 was used, while for the monoclonal antibodies tested, no more than 37% of the cells within PR+ BC samples were stained (**Table 2**). In agreement, the mean proportion score, which is use to rank from 1 to 5 the proportion of stained cells in samples throughout the studied population, obtained with the rabbit polyclonal antibody 62621 (4.8) was higher than with the two monoclonal antibodies, rabbit SP42 (4.2; $p<0.01$) and mouse 636 (4.3; $p<0.05$) (**Table 3**). On the other hand, there was no statistical difference between the mean proportion scores calculated for the two monoclonal antibodies (**Table 3**), a result corroborated by those of Rossi and colleagues [11]. Nevertheless, there are evidences pointing to higher sensitivity of the rabbit monoclonal antibodies, when compared to the mouse ones, in accessing PR in BC specimens [5, 23]. As aforementioned, we again noticed relatively good concordance between the two ant-PR monoclonal antibodies, rabbit SP42 and mouse 636 ($\rho=0.8588$), but poor concordance between SP42 and the rabbit polyclonal antibody 62621 ($\rho=0.4832$) as well as between 636 and 62621 ($\rho=0.4050$) (**Table 3**). Our data is in agreement with that of Press and colleagues [22], who tested 14 different antibodies for the detection of PR in BC and observed that of all the antibodies tested PgR636 and PgR1294 stained the highest percentage of breast carcinomas known to be positive by the biochemical assay (95-98%).

The Allred score (A-score), which ranges from 2 to 8, is calculated based on both the proportion of stained cells and the staining intensity of positive cases. As previously pointed out, the anti-PR monoclonal antibodies, rabbit 636 and mouse SP42, provided higher staining intensity in PR+ BC biopsies than the rabbit polyclonal antibody 62621; nonetheless, when the samples were probed with the latter the proportion of stained cells was higher than with the two monoclonal antibodies tested. Thereafter, the A-score of the three anti-PR antibodies evaluated in the present study were statistically similar (6.3 for SP42 and 62621; 6.6 for 636 - **Table 3**). It is worthwhile to point that the mean A-score for the three tested antibodies was statistically similar due to the compensatory effect of the high mean of proportion stained cells observed with 62621 on its low mean staining intensity. However, the apparent concordance among the three antibodies if only the mean A-score of the PR+ BC samples is taken into consideration is not in agreement with the other findings of the present work, neither is confirmed when we analyze the A-scores of each sample of the entire BC population studied (Data shown in **Table 2**). Indeed, when we expressed numerically the evaluation of the A-score obtained for each BC sample within the entire population evaluated, there was a lack of concordance between 62621 and SP42 (Spearman's correlation coefficient of 0.4365) and between 62621 and 636 (Spearman's cor-

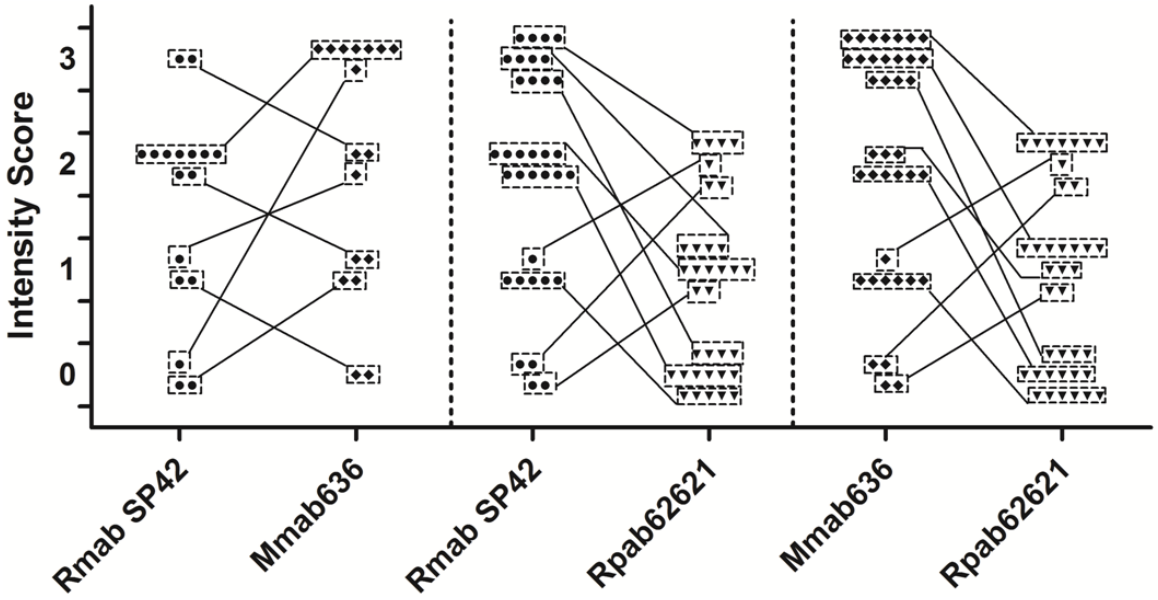

Figure 1. Discordance between intensity scores obtained with each antibody.

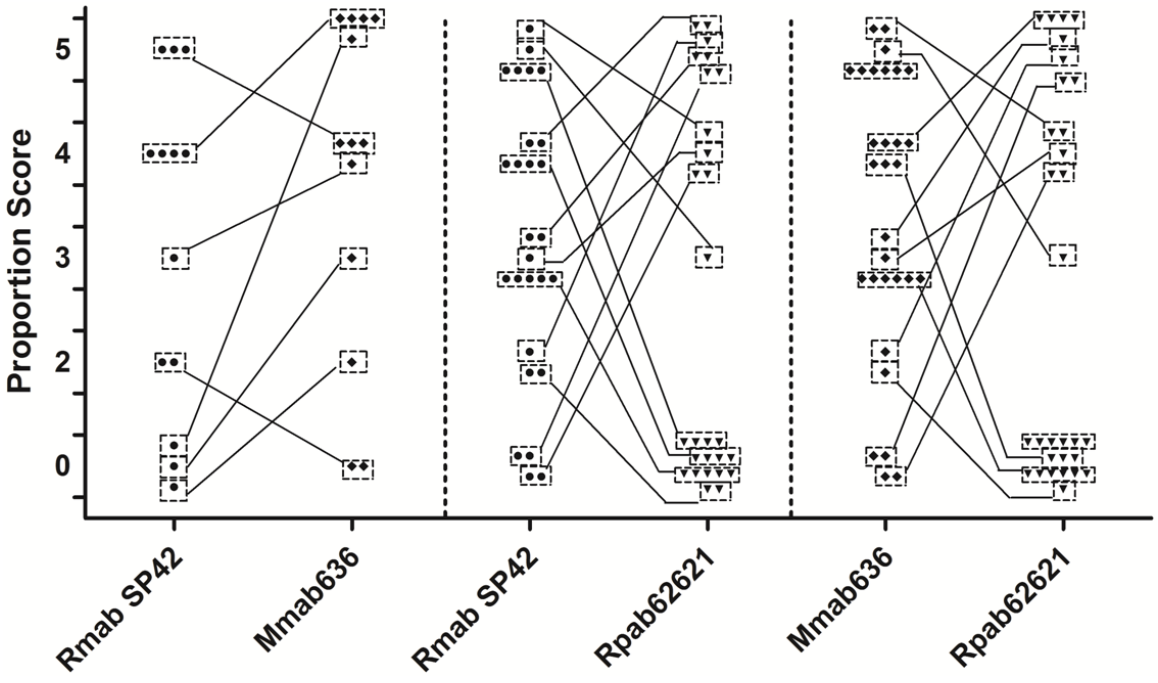

Figure 2. Discordance between proportion scores obtained with each antibody.

relation coefficient of 0.4062), but there was concordance between SP42 and 636 (Spearman's correlation coefficient of 0.8068).

We then inquired if the staining pattern and scoring were comparable among the whole BC population studied and each disease histological subtype. Unfortunately, due to the histological type distribution of our sample (**Table 1**), with high predominance of CDI cases, these were the only histological subtype in which we were able to perform statistics analyses. A consistent staining pattern and scoring was observed between the CDI cases and the entire BC population studied, that is, higher proportion of stained cells and PS, but lower intensity

score were obtained when the slides were probed with 62621 in comparison with SP42 and 636 (**Table 3**). Meaningful pointing out that although we did not have enough ISDC cases to get conclusive statistical analysis (n=6), a similar pattern tends to occur within the referred histological subtype (**Table 3**).

In order to better understand the inconsistencies among the studied antibodies, we realized a detailed analysis of the discordant results. Regarding the intensity score, in only 14 of the 53 tumor samples total concordance among the three antibodies was observed (7 negative, 2 weak, 3 moderate and 2 strong). Relatively low discordance (17 discordant cases) between the monoclonal antibodies 636 and SP42 was observed, while higher discordance was observed between SP42 and 62621, and between 636 and 62621 with 34 and 38 discordant cases, respectively (**Figure 1**). As for proportion score, total concordance was only observed within the RP-negative samples (7 cases) and samples with the highest score (12 cases), totalizing 19 concordant results. Once more, 636 and SP42 showed better concordance (13 discordant results) when compared to SP42/62621 and to 636/62621 (27 and 30 discordant cases, respectively - **Figure 2**). At last, only 10 samples were absolutely concordant for A-score, including 7 negative results. Again, as expected, better concordance was observed for 636 and SP42 (19 discordant results), while 39 discordant samples were observed when comparing the results obtained with SP42 and 62621, and 40 for those obtained with 636 and 62621.

Recently, scientists have explored to shed light on ER regulated genomic events in primary BC with divergent clinical outcome and in distant ERα+ metastases. Mapping of genome-wide ER binding events by chromatin immunoprecipitation followed by high-throughput sequencing revealed differential ERα-chromatin binding programming that results in predictive gene signatures exclusive for ERα+ breast cancer clinical outcome, and is characterized by remarkable intensification of ERα binding signal in tumors that progress toward a poor prognosis [24]. Furthermore, ERα-chromatin interactions occur regardless of tumor endocrine therapy sensitivity. Nevertheless, there is differentially stronger ERα binding signal in tamoxifen resistant in comparison with tamoxifen sensitive lineages [24]. Although the mechanisms underlying ERα binding plasticity in breast cancer remain to be elucidated, the influence of specific stimuli, as those triggered by growth factors pathways, may result in differential ERα binding patterns that regulate gene expression programs, sensitivity to endocrine therapy and overall clinical outcome in ERα+ breast tumors. The role of PR signaling pathways in the aforementioned events remain to be determined, however, there is a possibility that they might contribute to BC overall clinical outcome. This is reinforced by previous findings that have pointed PR- BCs as an aggressive form of the disease, partially due to increased expression of growth factors receptors and associated tamoxifen resistance [8].

In conclusion, the anti-PR monoclonal antibodies (636 and SP42) are, unlike the polyclonal antibody ab62621, highly sensitive and equally suitable for immunohistochemical evaluation of PR status in BC specimens. Therefore, we strongly suggest their clinical use during BC diagnosis; thus, enabling more precise therapeutic decisions to treat BC.

Acknowledgements

We acknowledge FAPES for Renata Dalmaschio Daltoe and Klesia Pirola Madeira doctorate scholarships. Assistance provided by Dr Curtis J. Henry was greatly appreciated.

Disclosure of conflict of interest

None.

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