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Molecular characterization of breast cancer cell lines by clinical immunohistochemical markers

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Abstract. Breast cancer is the leading cause of cancer mortality in females worldwide. Studies based on gene expression profiles have identified different breast cancer molecular subtypes, such as luminal A and B cells, cancer cells that are estrogen receptor (ER) and/or progesterone receptor (PR) positive, human epidermal growth factor 2 (HER2)-enriched cells, cancer cells that exhibit an overexpression of the oncogene *HER2*, and triple-negative cells, cancer cells that are negative for ER, PR and HER2 expression. Immunohistochemistry is the most common type of method used for the identification of these molecular subtypes, through the identification of specific cell receptors. The present study aimed to evaluate the ER, PR and *HER2* receptor expression in human breast cancer cell lines, and to classify the corresponding molecular subtype comparing two alternative methods. In the present study, a panel of human mammary carcinoma cell lines: BT-20; Hs578T; MCF-7; MCF-7/AZ; MDA-MB-231; MDA-MB-468; SKBR3; and T47D were used. Immunohistochemical and immunocytochemistry assays were used to characterize the breast cancer subtypes of these cell lines according to the expression of ER, PR and HER2 receptors. The results revealed the molecular characterization of this panel of breast cancer cell lines, using the differential expression of classical and clinically used markers in concordance with previous studies. In addition, these data are important for additional *in vitro* studies of these specific receptors.

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Introduction

Breast cancer is the second most common type of malignant neoplasm worldwide, and the most common type amongst females (1). The incidence of breast cancer in Brazil is similar to the incidence in developed countries, and varies according the regions, with the highest rates in southern region (2). A previous study demonstrated a decrease in female breast cancer mortality rates in the majority of developed Brazilian states, possibly due to an improvement of healthcare in these regions (3).

Despite advances in breast cancer clinics and research concerning care management, several questions remain. Breast tumors with similar histopathological appearances may exhibit different clinical presentations, levels of disease aggression and treatment responsiveness. The heterogeneity in breast cancer cell phenotypes and plasticity of the tumor microenvironment affects the therapeutic response and disease progression (4,5). At present, treatment options are based on the characteristics of the particular tumor, and treatment is multimodal (6-8). Therefore, a molecular approach by immunohistochemical evaluation is necessary to identify breast cancer subtypes for further treatment options (4,5).

Perou *et al* (9) initially classified breast tumors into four molecular subtypes according to their gene expression profiles: Estrogen receptor $(ER)^+$/luminal-like, receptor tyrosine-protein kinase erbB-2 (Erb-B2), basal-like and normal breast tissue. At present, due to the limitations of array analysis in clinical practice, particularly the high costs of this method, classification protocols have incorporated immunohistochemical classification techniques in clinical practice (10). There is clinical interest in distinguishing breast cancer subtypes, particularly the most aggressive types that exhibit poor prognosis, which possess a triple-negative profile that does not exhibit ER, progesterone receptors (PR) or the oncogene *HER2* (11-13). The differentiation of luminal B cells, which exhibit higher proliferation rates, from luminal A subtypes has a direct effect on the selection of treatment strategy (12,13). PR expression in >20% of tumor cells was suggested to increase luminal A sensibility (14), and the initial cut-off of the level of proliferation marker protein KI67 at ≥14% (10) that was initially proposed has been revealed to be increased to ≥20%, but this has not been confirmed (15).

Cancer cells *in vitro* are immortal and may exhibit spontaneously modified characteristics, so it is important to regularly characterize them. Although there are strong associations between particular types of cell line and specific immunohistochemical characteristics, they are not predictive and therefore it is important to translate *in vitro* data to a clinical context (16). The present study aimed to characterize the cell phenotypes of a panel of human breast carcinoma cell lines by measuring the expression levels of the markers classically used in clinic: ER, PR, HER2 and KI67, using IHC and immunocytochemistry (ICC). This data will be useful for subsequent *in vitro* studies investigating the expression of the specific receptors of the markers.

Materials and methods

Breast cancer cell lines. A total of 8 breast cancer cell (BCC) lines were used in the present study: BT-20; Hs578T; MCF-7; MCF-7/AZ; MDA-MB-231; MDA-MB-468; SKBR3; and T47D were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO_2 atmosphere at 37˚C. The cells were provided by Dr Rui M. V. Reis from Molecular Oncology Research Center at Barretos Cancer Hospital (Barretos, Brazil).

IHC and ICC assays. The IHC assays were performed using paraffin-embedded cell pellets obtained from 150 cm^2 flasks, fixed in 10% buffered formalin. The cell pellet was centrifuged at 1.027 x *g* for 5 min at room temperature, dehydrated in different concentrations of ethanol bath (50, 70, 95 and 100%), immersed in xylene baths for 2 min, and embedded in paraffin blocks to obtain 4-μm sections. The IHC process followed the standard automated process of the Ventana BenchMark Ultra automated slide staining system (Ventana Medical Systems, Inc., Tucson, AZ) using a ultraView 3,3'-diaminobenzidine (DAB) detection kit and prediluted primary antibodies from Ventana Medical Systems, Inc. with the following specificities: ER (cat. no., 790-4325; clone SP1), PR (cat. no., 790-2223; clone 1E2), HER2 (cat. no., 792991; clone 4B5) and Ki67 (cat. no., 790-4286; clone SP6).

ICC was performed as follows: The cells were seeded onto circular glass coverslips of 24 mm diameter at 80% confluence, fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) for 15 min at 4˚C, followed by permeabilization with 0.2% Triton X-100 for 4 min. Coverslips were placed on the slides, which were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase and incubated in a humidified chamber for 60 min at room temperature. Anti-ER, anti-PR and anti-HER2 were used as the primary antibodies using pre-dilution solutions as aforementioned. The slides were washed with PBS and incubated for 30 min at room temperature with the polyvalent secondary antibody from the UltraVision Quanto Detection System horseradish peroxidase DAB kit (cat. no. TL-125-QHD; Thermo Fisher Scientific, Inc.) and streptavidin-peroxidase (Thermo Fisher Scientific, Inc.) for 10 min using a DAB chromogenic kit (Agilent Technologies, Inc., Santa Clara, CA, USA).

The ER and PR staining reactions were scored according to nuclear intensity, weak, moderate and high, and extension through the following criteria: 0%, negative; 1 to 25%, low expression; 26 to 50%, moderate expression; 51 to 75%, high expression; above >75%, very high expression. HER2 expression was analyzed according to the American Society of Clinical Oncology (ASCO) /College of American Pathologists HER2 test guidelines (17).

Results

The expression level of specific receptors in the BCC BT-20, Hs578T, MCF-7, MCF-7/AZ, MDA-MB-231, MDA-MB-468, SKBR3 and T47D lines, was assessed by IHC (Fig. 1) and ICC (Fig. 2). Despite differences with respect to expression intensity between the two techniques, similar results regarding ER, PR and HER2 expression were observed.

The staining intensity of the cell lines tested using IHC is represented in Fig. 3, which revealed the BCC subtype characterizations as i) MCF-7, MCF7-AZ and T47D, Luminal; ii) BT-20 and SK-BR3, HER2 overexpressed and; iii) MDA-MB-231, MDA-MB-468 and Hs578T, triple negative subtype.

Discussion

Breast cancer is a heterogeneous disease characterized by significant variability in morphological and pathological features that may exhibit differences regarding therapeutic responses and disease progression, which significantly affect the management of this disease (5). Perou *et al* (9) initially classified breast tumors into four molecular subtypes according to their gene expression profiles: ER^+/luminal-like, HER2, basal-like and normal breast. This molecular classification has been altered to five categories: Luminal A, Luminal B+C, normal-like, basal like and $ErbB2^+$, based on prognostic evaluation (18). Recently a novel subtype classified as claudin-low has also been identified based on microarray gene expression platform (19).

IHC has been applied as a diagnostic method to identify the five classical subtypes of breast cancer based on immunohistochemical profiles: Luminal A, ER^+ and/or progesterone receptor positive, $HER2^-$; luminal B, ER^+ and or PR^+, $HER2^+$; basal-like, $ER^-PR^-HER2^-$, cytokeratin $5/6^+$, and/or $HER1^+$; $HER2^+/ER^-$, ER^-PR^- and $HER2^+$; and unclassified, negative for all five markers. A strong correlation was observed between the molecular marker profiles and immunohistochemical evaluation (20). HER2 tumors are immunohistochemically categorized as +++/+++ or when fluorescence *in situ* hybridization demonstrates *HER2* gene amplification (17,21). The most recent ASCO/CAP recommendation for HER2 testing changed the threshold for determining HER2-positive status with immunohistochemistry to strong circumferential membranous staining in ≥30% of the tumor cells, whereas the previous threshold was ≥10% (17), which may cause discrepancies between studies. The Ki67 index was added to immunohistochemical evaluation, and the labeling index of 13.25% separates luminal $A/HER2^-$ and luminal B/HER^- tumors, with a sensitivity and specificity of 72 and 77%, respectively, for gene expression (22). As it is not cost-effective to perform individual

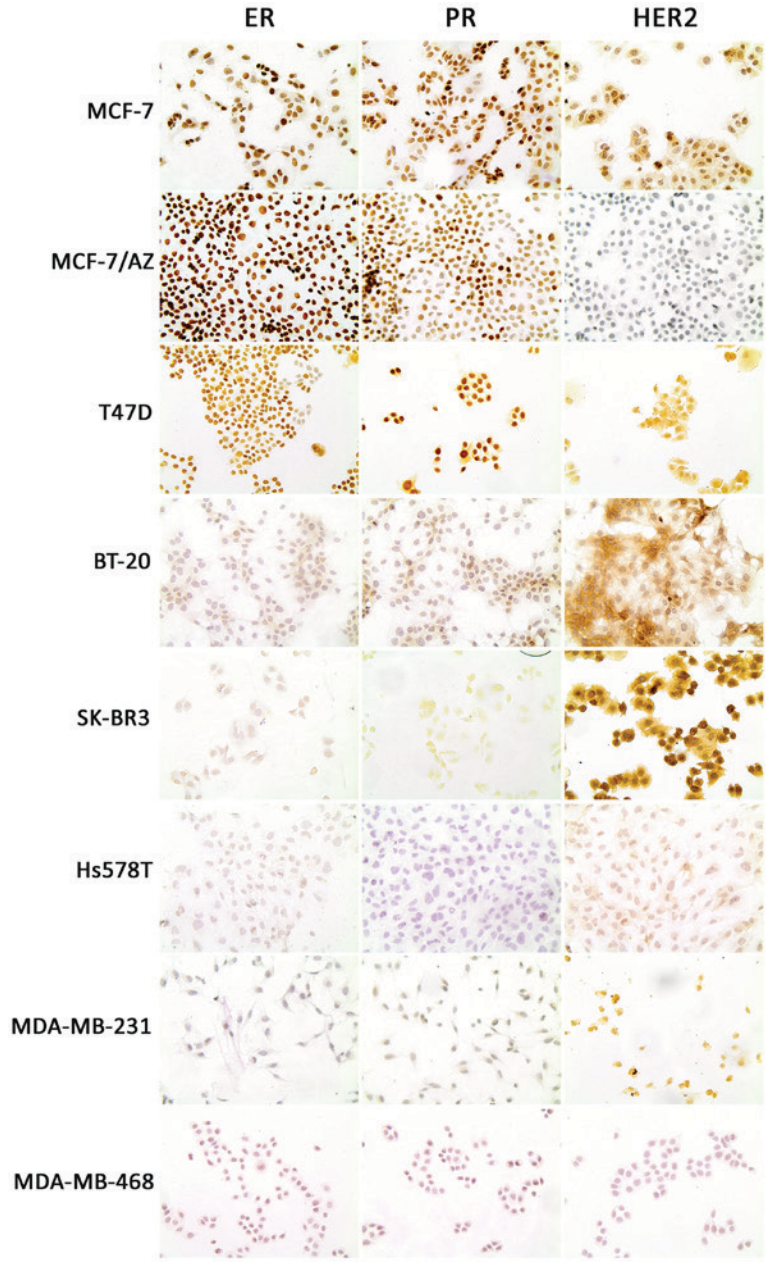

Figure 1. Representative immunohistochemistry for ER, PR, and HER2 in breast cancer cell lines MCF-7, MCF-7/AZ, T47D, BT-20, SKBR3, Hs578T, MDA-MB-231 and MDA-MB-468. Scale bar=100 μM. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor.

molecular classifications in clinical practice, the 12th St Gallen Consensus (10) was adopted, and comprises 5 classifications based on immunohistochemical results: Luminal A, Ki67 <14; Luminal B HER$^-$, Ki67 ≥14; Luminal B Her$^+$; HER2 positive, non-luminal and basal-like, triple-negative. Prat *et al* (14) suggested the inclusion of PR positive tumor cells ≥20% to increase luminal A sensitivity Novel amendments to classification protocols have been proposed to include the level of KI67 expression: High expression, ≥20%; intermediate expression, 14-19%; PR negative or low expression, <20%, but this has not been formally adopted (15). The present study aimed to characterize ER, PR, Ki67 and HER2 receptor expression in BCC lines to classify the corresponding molecular subtype (10).

According to the gene expression profiles, breast tumors may be classified into luminal A, luminal B and HER2-overexpressed and triple-negative subtypes (9). The expense of this classification system has limited the incorporation of gene expression profiling into clinical practice, and therefore, IHC assays are used for the identification of molecular subtypes, which may characterize the gene expression profiles of the cells through ER, PR and HER2 receptors (13). The differences observed in the marker intensity between the two techniques did not affect the classification of the molecular subtypes of the cell lines.

The classification of BCC lines includes the presence of ER on luminal A and B, HER2 overexpression of *HER2* oncogene on HER2 subtype, and absence of ER expression, PR and HER2 on triple-negative cells. Although certain luminal B tumors may be identified by their expression of HER2, the distinction between luminal A and B relies on the observation of the rate of proliferation, including the level of expression of Ki67 in the luminal B subtype (9,12,23). In the present study,

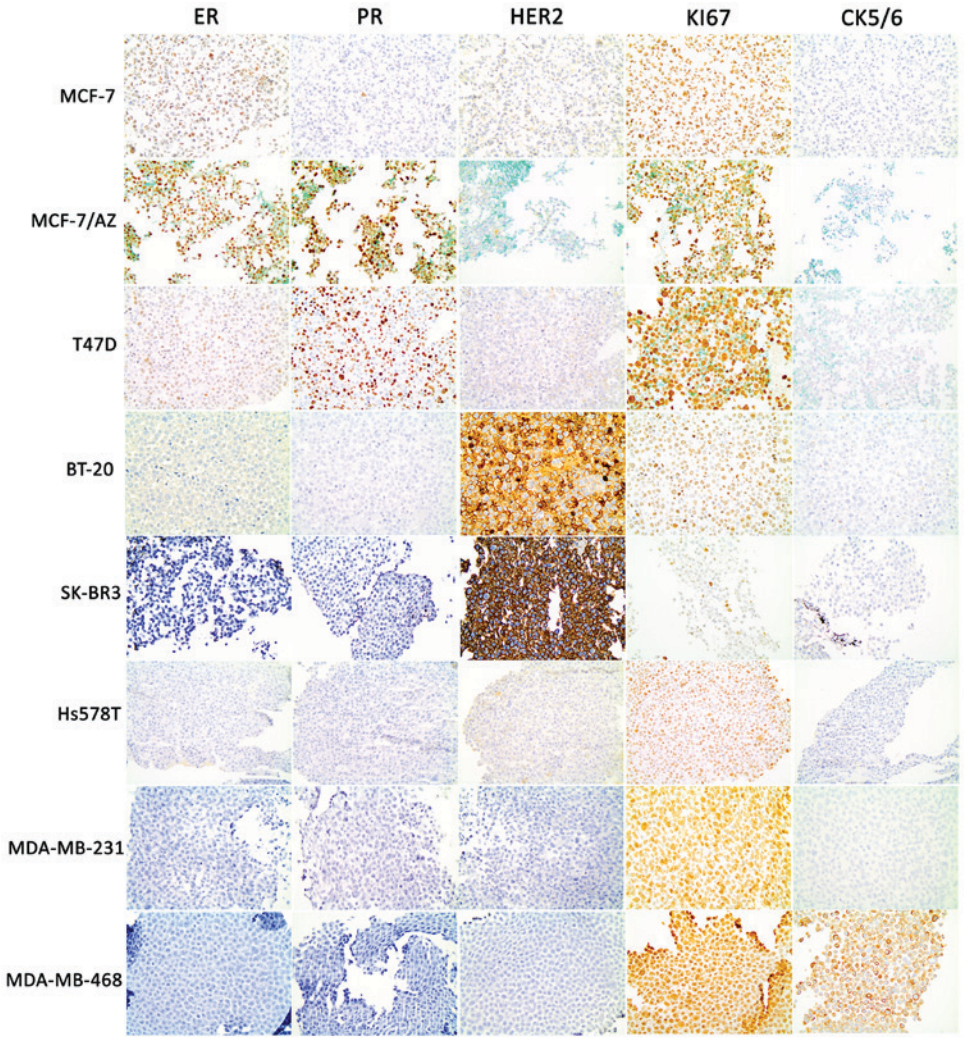

Figure 2. Representative immunocytochemistry for ER, PR, HER2, KI67 and CK5/6 in breast cancer cell lines, MCF-7, MCF-7/AZ, T47D, BT-20, SKBR3, Hs578T, MDA-MB-231 and MDA-MB-468. Scale bar=100 μM. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor; CK, cytokeratin.

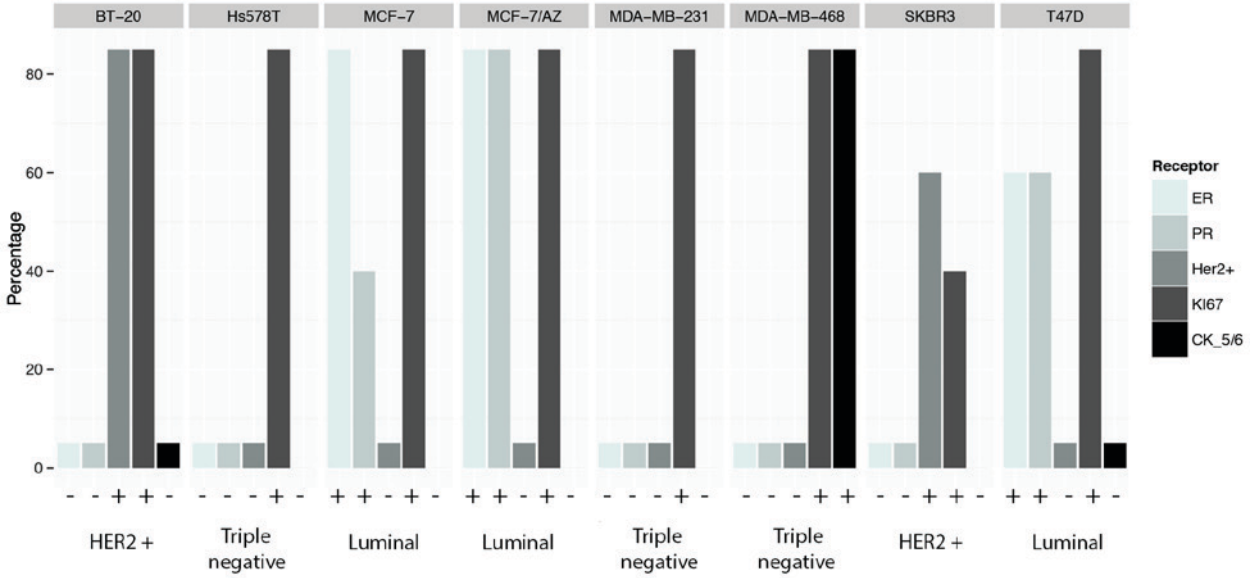

Figure 3. Staining intensity scores of receptor markers ER, PR, HER2, Ki67 and CK5/6 expression, obtained by immunohistochemistry and immunocytochemistry in breast cancer cell lines BT-20, Hs578T, MCF-7, MCF-7A/Z, MDA-MB-231, MDA-MB-468, SKBR3 and T47D. Luminal, ER^+/PR^+; $HER2^+$, HER2 overexpressed; triple-negative, does not exhibit ER, PR or HER2. ER, estrogen receptor; PR, progesterone receptor; CK, cytokeratin; HER2, human epidermal growth factor.

it was not possible to observe a difference between luminal A and B, possibly due to changes in the levels of expression of some proteins observed on longer culture conditions. However, the general classification is in concordance with American Type Culture Collection and associated studies (24).

Subik *et al* (16) performed an IHC analysis of 17 breast cancer cell lines, using ER, PR, HER2, cytokeratin (CK) 5/6, cytokine and growth factor (CGFR), Ki-67 and androgen receptor (AR) as markers, although did Ki-67 and AR were not used for the classification, and tumors were considered HER2-positive only when this protein was highly expressed. The authors used the Carey initial classification (20), and observed a high level of Ki-67 in this panel of breast cancer cell lines. The aforementioned study considered the importance of evaluating the *in vitro* cell characteristics, but did not compare the molecular classification with the final results observed in the immunohistochemistry analysis.

BCC lines are used as *in vitro* models to investigate gene and protein expression, responses to drugs and for toxicity assessments. These cells must be characterized for quality control, and to provide better reproducibility of studies based on the expression of specific receptors. The results of the present study demonstrate that the characterization of the molecular subtypes of BCC lines by IHC and ICC assays is possible, but that there is not complete concordance, suggesting there is a potential limitation on the immunohistochemistry classification or a false-negative result.

In conclusion, the present study characterized the phenotype of a panel of BCC lines, according to their ER, PR and HER2 markers. This data may provide a tool for an increased understanding of cellular behavior, and confirm knowledge from prior *in vitro* studies based on cell receptor expression. The present study demonstrates similarities between ICC and IHC techniques, which may affect local quality control protocols for these cells.

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