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Immunohistochemiluminescence detection: A quantitative tool in breast cancer HER-2 status evaluation

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Abstract. Her-2 status evaluation in breast cancer has prognostic and treatment response value but its interobserver variation among pathologists is a problem since it is not quantitatively assayed. This study presents an immunohistochemiluminescence method to quantify Her-2 in breast cancer. Anti-Her-2 antibody was conjugated to acridinium ester (AE) and used to evaluate/quantify Her-2 status in breast Invasive Ductal Carcinoma (IDC, $n = 50$) comparing with traditional immunohistochemistry. Anti-HER-2-AE results were expressed in Relative Lights Units (RLU) and showed to be able to distinguish and quantify the differences between the three groups of Her-2 status. 3+ Her-2 status presented the highest RLU (246,982 $\times$ 10^3 $\pm$ 2.061 $\times$ 10^3) compared to 2+ (76,146 $\times$ 10^3 $\pm$ 0.290 $\times$ 10^3), negative (27,415 $\times$ 10^3 $\pm$ 1.445 $\times$ 10^3) and normal tissues (27,064 $\times$ 10^3 $\pm$ 2.060). Status differences were significant between 3+ and 2+ ($p = 0.0025$); 2+ and negative ($p = 0.0003$), and +3 and +1 ($p = 0.0001$) beside this, normal breast control RLU was 27,064 $\times$ 10^3 $\pm$ 2,060 $\times$ 10^3, similar to negative cases. Results showed that anti-HER-2-AE conjugate was effective in breast tumors Her-2 status evaluation, allowing its quantitative establishment to consequently decrease the subjectivity in prognostic and predictive information intrinsic to this test.

Keywords: Immunochemiluminescence, HER-2, invasive ductal carcinoma

1. Introduction

Chemiluminescence (CL) is described as a production of electromagnetic radiation from a chemical reaction, where the CL intensity is dependent on the concentration of a reagent participating in the chemiluminescent reaction [1]. This technique has been widely used in several areas of analytical applications due to its ultra-sensitive detection ranging from attomole to zeptomole in short-term assays [2].

Acridinium ester (AE) CL has been studied around four decades [3] and has been considered an effective marker to biomolecules signaling [4]. AE is used in chemiluminescent immunoassays as an alternative to luminol or isoluminol and even for enzyme based immunoassays with horseradish peroxidase because of its good half-time and easy quantification [5].

Molecular biomarkers, such as Her-2, play key roles in the diagnosis, prognosis and predictive value of many cancers, having great importance in monitoring the clinical course of disease and therapy response [6]. Her-2 over expression in invasive ductal carcinoma (IDC) characterizes a more aggressive tumor growth

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and a poor prognosis due to the increase in the signaling of cell growth and survival [7]. This oncogene is amplified in 10 to 30% of patients with IDC and it is considered a major prognostic factor also being used as an immunotherapy treatment indicator [8].

Immunohistochemistry is one of the most common choices for diagnostic, prognostic and predictive markers evaluation but it still has its limitations. Its semiquantitative analysis nature and the groups' categorization in null or weak, moderate and intense immunostaining, besides researcher's experience, should be taken account in the final result [9,10]. In this scenario, chemiluminescence provides quantitative results minimizing subjectivity [11]. So, the present study aimed to obtain anti-Her-2-EA conjugate and to evaluate their recognition assessment of Her-2 human breast cancer status in an immunohistochemiluminescence assay.

2. Materials and methods

2.1. Samples

Fifty formalin-fixed and paraffin-embedded biopsies diagnosed as invasive ductal carcinoma (IDC) and three normal tissues (from reduction mastoplasty) were obtained from Anatomy and Pathology Service of University Hospital at Federal University of Pernambuco (UFPE), Brazil. This study was approved by the Health Science Centre Bioethical Board from Federal University of Pernambuco, Brazil CEP/CCS/UFPE No 195/09.

2.2. Immunohistochemistry

Biopsy slices (4 μm) were deparaffinized in xylene, and followed by hydration with graded ethanol (100%–70%). Endogenous peroxidase blocking was performed using methanol-hydrogen peroxide solution, followed by antigen retrieval in 10 mM citrate buffer pH 6.0 in water-vapor chamber. After this samples were incubated with anti-HER-2 (1:400) primary antibody for 2 h at 4°C, and then with biotin-free polymer for 45 min at room temperature (both from DAKO®, USA). Reaction was visualized with diaminobenzidine (DAB) and tissues were counterstained with haematoxylin. Staining was evaluated by optical microscopic analysis. Protocols were carried out in triplicate, and subsequently analyzed by two pathologists. Negative controls were obtained replacing the primary antibody for mouse IgG isotype (DAKO). Immunohistochemistry reactions for Her-2 were scored by HercepTest™ where 0 and 1+ scores are negative, 2+ is weak positive and 3+ is positive [9]. Currently the College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO) suggested that the 2+ score were considered indeterminate, recommending the use of a more specific test as fluorescence *in situ* hybridization (FISH) for HER-2 status classification [12,13]. In our samples, we used the HercepTest™ system and adopted the CAP/ASCO recommendation for 2+ samples to be considered indeterminate.

2.3. Anti-Her-2-AE conjugation

Anti-Her-2 was conjugated to AE according to Campos et al. [2]. The antibody (1 mL containing 1.1 mg of protein) was incubated for 1 hour at 25°C with 7 μL of AE solution (0.2 mg diluted in 400 μL of N,N-dimethylformamide). The anti-HER-2-AE conjugate was applied to a Sephadex G-25 column (10×1 cm), previously equilibrated with 100 mL of 10 mM phosphate buffer, pH 7.2 (PBS). Aliquots (1 mL) were collected, protein content was determined at 280 nm, and their CL assayed. Aliquots of protein peak with chemiluminescence were pooled and kept at 4°C until use.

2.4. Immunohistochemiluminescence using Anti-HER-2-AE

IDC biopsies were cut (8 μm) and deparaffinized with xylene followed by hydration with graded ethanol. Slices were incubated with 100 μL of Anti-Her-2-AE (48 μg/mL) conjugate for 2 hours at 4°C and then tissue areas (1 cm^2) were transferred to separate Eppendorf tubes containing 50 μL of PBS. Chemiluminescence was measured in a Luminometer Modulus Single Tube 9200-001 (Turner BioSystems, USA) adding 50 μL of reagent A (hydrogen peroxide) and 50 μL of reagent B (sodium hydroxide). Foton emitions were measured as relative light units (RLU). Samples were assayed in triplicate and the relationship between RLU x tissue area (0.125 to 1 cm^2) was assayed also in triplicates.

2.5. Statistical analysis

Mann Whitney test was performed in GraphPad Prism 5 software. Differences were considered significant when $p < 0.05$. In all graphs, bars represent mean value + standard deviation.

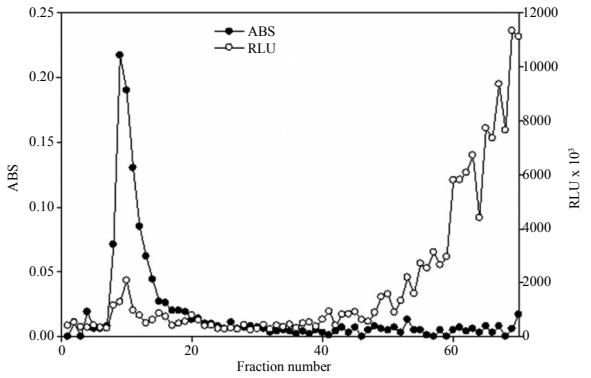

Fig. 1. Anti-Her-2-AE elution profile from Sephadex G-25 column (10×1 cm). Elution was carried out with 10 mM phosphate buffer (PBS), pH 7.2. Aliquots (1 mL) were collected and chemiluminescence and protein content assayed.

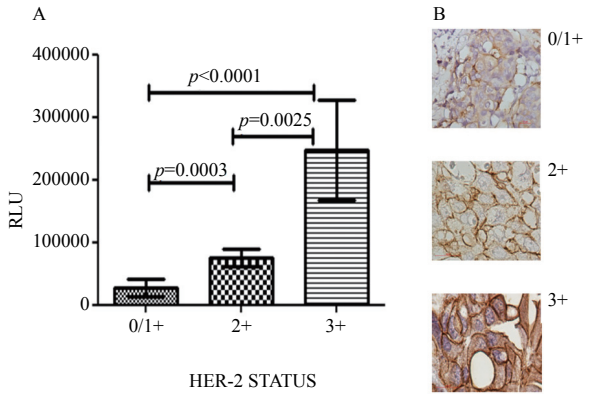

Fig. 2. (A) Chemiluminescence profile of anti-Her-2-AE (B) Immunohistochemistry of IDC samples. 0/1+ (400× magnification), 2+ (1000× magnification) and 3+ (1000× magnification). (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-130981)

3. Results

Initially traditional IHC tests were performed and showed that 7 of 50 patients samples were 3+ for Her-2, five cases were indeterminate (2+) and 38 were negative (1+ or 0). Purification of anti-Her-2-AE conjugate was carried out in Sephadex G-25 column and its profile is shown in Fig. 1. After purification the conjugated was used as an immunohistochemiluminescence probe showing significant difference ($p = 0.0025$) in RLU emitions between 3+ cases ($246,982 \times 10^3 \pm 2.061 \times 10^3$) and 2+ cases ($76,146 \times 10^3 \pm 0.290 \times 10^3$) as well as between 2+ and negative samples ($27,415 \times 10^3 \pm 1.445 \times 10^3$), $p = 0.0003$. 3+ and 1+ cases also presented a significant difference, $p < 0.0001$ (Fig. 2). The RLU for normal breast control was $27,064 \times 10^3 \pm 2,060 \times 10^3$, similar to negative cases analyzed.

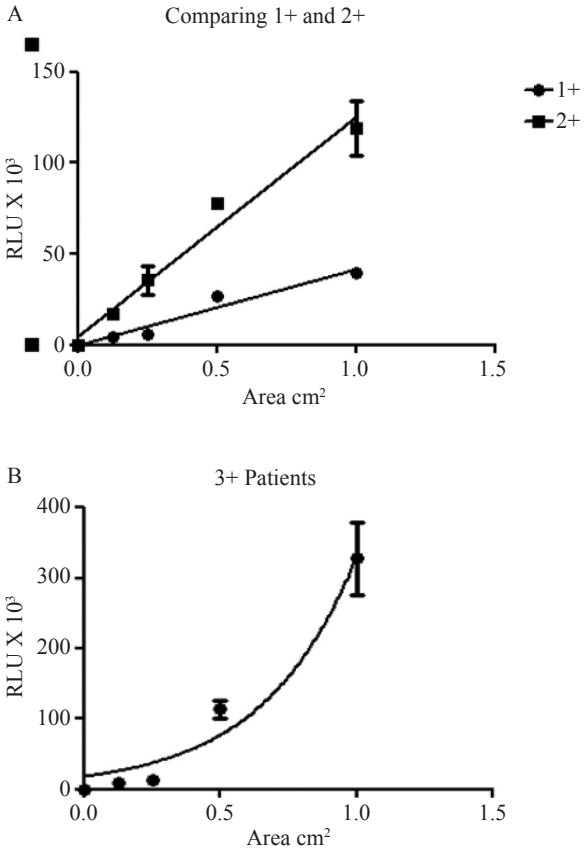

Fig. 3. Tissue area versus RLU: (A) linear relationship between 0/1+ ($r^2 = 0.9517$) and 2+ ($r^2 = 0.9743$) samples; (B) exponential relationship for 3+ samples.

RLU from Her-2-AE revealed that light emission was a tissue-area-dependent. The proportional increase in RLU with tissue area, observed in all groups analyzed, is related to increase in the amount of antigen-antibody complex formation in tissue areas evaluated (Fig. 3).

4. Discussion

In the last decades it has been observed an increasing incidence of breast cancer with growing mortality rates [14]. Today immunohistochemistry (IHC) is the most used method to evaluate prognostic and therapeutic response including Her-2 status due to its relatively low cost [15]. In clinical practice, Her-2 overexpression is considered a predictive marker to determine which patients could be benefited with hu-

manized monoclonal antibody (Trastuzumab®) treatment which has a satisfactory response to targeted therapy [16]. The use of Trastuzumab® as monotherapy or in combination with other drugs has significantly improved progression, overall survival and life quality rates of early stages breast cancer patients [16,17]. Due to the Trastuzumab® treatment benefits Her-2 status has always been requested by oncologists but the two techniques approved by the US Food and Drug Administration (FDA), IHC and FISH, still have limitations such as low reliability and a high cost [16,18]. The high cost of this drug has fueled the determination of new guidelines for diagnosis [18]. Due to the intrinsic results subjectivity of both techniques, the search of new methods with less subjectiveness needs to be developed.

Chemiluminescent assays have been widely used as a quantitative assay in recent years [19]. The method has great advantages such as high sensitivity and stability of reagents, biological low-risks, easy-to-use protocols and detection accuracy, non-photodegradable products and no background [5,20]. In our study we propose an immunohistochemiluminescent method to quantify Her-2 status in breast tissues (normal and diagnosed as invasive duct carcinoma, IDC) and to compare to traditional IHC. For our pleasure the results showed reliability and relative low cost indicating the same as a new tool for laboratory analysis in Her-2 detection. Our findings showed that 14% of samples were 3+ for Her-2 in IHC, 10% indeterminate (2+) and 76% were negative (1+ or 0). The 14% of Her-2 positivity observed in our study is consistent with frequency found in others breast cancer studies [8].

In this study anti-Her-2-AE was evaluated as an immunohistochemiluminescence probe, and for our pleasure, showed significant difference ($p = 0.0025$) in RLU emitions between 3+ cases and 2+ cases as well as between 2+ and negative samples ($p = 0.0003$). 3+ and 1+ cases also presented a significant difference ($p < 0.0001$). Weeks firstly reported the use of AE conjugated antibody as an immunochemiluminescence detecting method for α-1-fetoprotein [21] but in our lab studies using Concanavalin A (Con A – glucose/mannose specific), conjugated to AE established that there is a difference in RLU between normal and IDC samples [2]. In another study we showed that Con A-AE RLU for IDC was lower than to fibroadenoma (a benign breast disease) [19].

RLU from anti-Her-2-AE revealed that light emission was a tissue-area-dependent. These results indicate that tissue area is an important parameter in the histochemiluminescent reaction rate under the present conditions when HER-2-AE is used as histochemistry probe. The standard deviation observed in area analysis should be a reflect of the HER-2 antigen expression and/or disposition/accessibility to their moiety in cell membrane.

Patients who present IDC with HER-2 over-expression have more aggressive tumor progression, a high degree of malignancy, metastasis and poor prognosis [7, 22,23]. In our study positive cases (3+) showed an exponential increase in RLU indicating a high degree of malignancy (Fig. 3). Histopathology data of these patients included positivity to lymph node metastases and a decrease in overall survival and disease-free survival [24]. In negatives or (1+) and indeterminate (2+) cases it was observed a linear increase in RLU profile, regarding tissue area, indicating less antigen availability when compared to 3+ patients. These results are in accordance with our previous studies [2,19] confirming the effectiveness of this technique comparing to histochemistry procedures.

Our results indicate a correlation between IHC and immunohistochemiluminescence results, where 3+ patients exhibited the highest RLU. Analysis of RLU and tissue area demonstrated that area is an important parameter for chemiluminescence detection under the established conditions in our study using anti-Her-2-AE conjugated. These results provide satisfactory data and contribute to the quantification of cell surface antigens present in IDC tumors being immunohistochemiluminescence an important tool in prognostic and predictive biomarkers analyses in breast neoplasia.

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