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Correlation and comparison of immunohistochemistry for HER2/neu, using the antibody *SP3* and chromogenic *in situ* hybridization in breast carcinomas samples

Correlação e comparação de imuno-histoquímica para HER2/neu, utilizando o anticorpo SP3 *com a hibridização* in situ *cromógena em amostras de carcinomas mamários*

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ABSTRACT

Introduction: Advances in the field of molecular biology have provided the differentiation of molecular subtypes of breast tumors, providing better prognosis and important tools for the treatment of patients with breast cancer. Among these subtypes, the changes in the human epidermal growth factor receptor 2 gene (*HER2/neu*), increase its copy number and generating HER2 protein amplification. Studies show that patients with breast cancer HER2/neu amplified tend to relapse earlier and have shorter survival time, the monoclonal antibody *Trastuzumab* is the therapy indicated. The eligibility of patients for therapy is initially made by the immunohistochemistry (IHC) technique, which evaluates the expression level of the HER2 protein. After this evaluation, the cases with equivocal diagnosis (score 2+), are referred to a more accurate technique, the chromogenic *in situ* hybridization (CISH). **Objective:** To analyze the sensitivity and specificity of the antibody *SP3*, and determine their level of agreement with the CISH technique. **Material and methods:** Retrospective study in the database of the anatomy-pathology laboratory, in CISH tests reports for HER2/neu. **Conclusion:** The results revealed that clone *SP3* showed 100% specificity and 92% sensitivity. IHC reveals variability in its results; however, it is known that the technique is an important tool in the daily routine of laboratories, contributing to the initial screening of patients with breast cancer, which later showed satisfactory results when compared with the CISH technique.

Key words: breast cancer; *in situ* hybridization; immunohistochemistry.

INTRODUCTION

Breast cancer is among the most common neoplasm affecting women, both in developing and developed countries[1, 2]. In Brazil, according to the Instituto Nacional de Câncer (INCA), it were estimated for the years 2014 and 2015, about 57,000 new cases of female breast cancer each year, making it the second most frequent in the female population (non-melanoma skin cancer is the first one). It ranks third among cancers that affect the population in general (first non-melanoma skin cancer and second prostate cancer)[1].

Breast cancer is a heterogeneous disease, with a variety of morphological appearance and molecular characteristics. Studies in the field of molecular biology have provided the differentiation of five molecular subtypes of breast cancer: 1) luminal A; 2) luminal B-HER2 negative; 3) luminal B-HER2 positive; 4) basal-like or triple negative; and 5) HER2 overexpressing. They assist in understanding the mechanisms that regulate differentiation and cell proliferation, and thus allowing a better prognosis that provides important tools for the choice of therapy[2, 3].

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LITERATURE REVIEW

Human epidermal growth factor receptor-2 genotype (*Her2/neu*)

The *HER2/neu* gene is variously known as *neu*, *HER2* and *c-erb-B2*. A mutated version of the gene was first observed in a rat neuro glioblastoma and, therefore, named neu[4]. The *HER2/neu* comprises breast carcinoma with estrogen receptor (ER) and progesterone receptor (PR) negative, but that present overexpression of the protein also called HER2[2, 5]. In normal cells and in the majority of breast cancers, only two copies of the gene expressing low levels of p185 protein (protein HER2) are usually transported[6]. The gene amplification or overexpressing is evaluated on 25% to 35% of invasive breast carcinomas[7, 8], which, in most cases (around 90%), are attributed to the amplification of this protooncogene located on long arm of chromosome 17 (17q12)[5, 7, 9, 10]. A minority of cases where it is observed *HER2/neu* gene amplification is characterized by chromosome 17 polysomy, which leads to increased gene copy number, resulting in high expression and thus providing equivocal diagnostic[9].

There are reports in the literature on patients with breast cancer with amplified *HER-2/neu*, they tend to have early recurrence, and reduced survival[11]. The therapy with humanized monoclonal antibody *Trastuzumab* combined with adjuvant chemotherapy was initially used to prolonging survival in patients with *HER2/neu*-positive metastatic breast cancer. Moreover, it was also indicated to reduce the chances of developing distant metastases in patients who do not already have them[7, 12]. This benefit occurs because the treatment is directed to the antigen HER2/neu protein, inhibiting the growth of tumor cells with *HER2/neu* overexpressing[13]. The correct detection of *HER-2/neu* oncogene amplification is essential to enable eligibility of patients with breast cancer and lead them to treatment with *Trastuzumab*. This product is indicated because it is a specific targeted therapy, which acts blocking the extra cellular domain of receptor[12, 14, 15].

Protein overexpressing and immunohistochemistry (IHC)

IHC is a technique used to analyze the overexpression of p185 protein in tissue of mammary carcinomas, and was incorporated to surgical pathology as a complementary method. It is considered a technique of low cost, besides being the most used in the daily routine of pathology laboratories[14, 16]. The development of monoclonal antibodies provided great source of highly specific reagents for the demonstration of different tissue and cell antigens; along with the advent of antigen retrieval they were considered milestones in the evolution of IHC[17].

Several mono and polyclonal antibodies are commercially available for use in IHC, with differences in sensitivity and specificity, and may result in variations in the final quality of the reactions[18]. Among these antibodies options commonly used in the method, the mouse monoclonal antibody *CB11* and rabbit monoclonal antibody *SP3* stand out.

The principle of the IHC method lies in binding primary antibodies that recognize the specific antigen. The limitations associated with the Avidin-Biotin complex system led to the development of systems detection with enhanced sensitivity and specificity, using a polymer-based IHC method. This method uses a polymer structure with multiple secondary antibodies and enzyme molecules conjugated[19, 20] (**Figure 1**).

IHC provides the results of protein expression of the *HER2* gene using a scoring system ranging from zero to three[18]. According to the guidelines of the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), the staining intensity of the cytoplasmic membrane must be observed, which has values ranging from negative (0), no immunoreactivity or immunoreactivity ≤ 10% of the tumor cells; negative (1+), weak or faint immunoreactivity in more than 10% of the tumor cells, but only part of the membrane is positive; equivocal (2+), weak to moderate complete membrane immunoreactivity in more than 10% of the tumor cells or intense circumferential membrane staining in ≤ 30% of the cells; and positive (3+), moderate to strong complete membrane immunoreactivity in more than 30% of the tumor cells[15, 21-23]. The use of this scale has varying interpretations that depend on the technique reaction quality, the type of antibody used, and the observer evaluation[18]. These discrepancies in interpretations of the results are, especially when the IHC revels score 2+, and this value was considered inconclusive

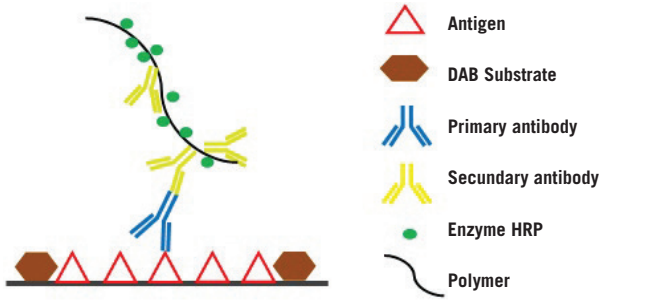

FIGURE 1 – *Representation of IHC reaction*

IHC: immunohistochemistry; DAB: diaminobenzidine; HRP: horseradish peroxidase.

for diagnosis. Based on this, the samples are sent to be tested *in situ* hybridization (ISH), since they are able to provide accurate and reliable results[18, 24].

Overexpression of gene and ISH

The ISH techniques determine the number of copies of a gene. They use complementary deoxyribonucleic acid (DNA) probes, marked to the target genomic sequencing. Regarding the patients with breast tumors, the chromogenic *in situ* hybridization (CISH) technique assesses the level of amplification of the *HER2/neu* gene, measuring its copy number by combined ISH, as well as by a digoxigenin (DIG)-labeled probe, which targets the *HER2* gene, and a dinitrophenol (DNP)-labeled centromeric probe, specific for the centromeric alpha-satellite region of chromosome 17[25] (**Figure 2**). The formation of double-stranded marked with probing may be visualized using primary antibodies (unlabelled), which are detected by secondary antibodies conjugated to polymerized enzyme. The enzymatic reactions of the substrates lead to the formation of strong red signals (chromosome 17) and green (*HER2* gene) permanent[25]. This reaction creates a chromogenic reaction similar to IHC staining, which enables viewing by light microscopy, as well as to avoid the need for fluorescence (used in the ISH method by fluorescence [FISH]), and provide a permanent staining record result. In addition, CISH has a lower cost compared with the fluorescent method and is more easily interpreted by pathologists, since it can be correlated with tumor histology at the same time because they are more used to IHC labeling than the fluorescent signal[6, 15, 16, 24].

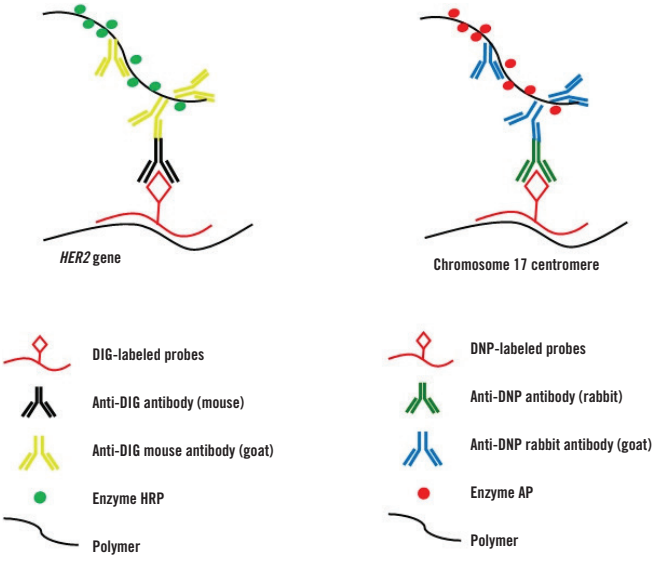

FIGURE 2 – *Representation of CISH reaction*

CISH: chromogenic in situ *hybridization; DIG: digoxigenin; HRP: horseradish peroxidase; DNP: dinitrophenyl; AP: alkaline phosphatase.*

CISH has emerged as a potentially promising alternative to FISH, showing high correlation between its results in the literature[6, 15, 16, 26, 27]. Both methods are approved by the Food and Drug Administration (FDA) and considered the gold standard to assess the status (level) of gene amplification when required for confirming the ambiguous results of IHC[16, 28]. Furthermore, CISH allows morphological tumor analyzing (interpreting the heterogeneity of it), the gene copies number of in different parts of the tumor and the observation of invasive or *in situ* carcinoma fields, providing a special clinical meaning to *HER2/neu*[16, 26-28].

OBJECTIVE

This study aimed to analyze the *SP3* antibody sensitivity and specificity and to determine their level of agreement with the CISH technique.

MATERIAL AND METHODS

This study was conducted through a survey of the Centro de Diagnóstico Anatomopatológico (CEDAP) database, located in the city of Joinville, Santa Catarina. This work is approved by the Research Ethics Committee of the Pontifícia Universidade Católica do Paraná (PUCPR) under CAAE record: 12717213.5.0000.0020. In the routine, the breast cancer samples are initially submitted to *HER-2* gene status search by IHC, using the *CB11* clone (Cell Marque®). When the result of this reaction presents score 2+, the assistant doctor responsible for the patient requests the CISH study (ZytoVision®) to definition the case. For ratification of the results of the CISH, a new IHC is requested now using *SP3* clone (Cell Marque®). In some special cases, the CISH research is still required by the assistant doctor, even when satisfactory results by IHC technique are revealed (score 0, 1+ or 3+).

Thus, we selected all reports from January 2011 to March 2014 that showed research *HER2/neu* gene amplification obtained by CISH technique, accompanied by IHC tests with *SP3* clone, totaling 98 cases.

The parameters for interpretation of IHC score and evaluation of gene amplification by CISH followed the recommendations of the ASCO/CAP, as shown in **Tables 1** and **2**, respectively[29]. The assessment performed by CISH has specific probe for marking the chromosome 17, excluding the chances of misinterpretation of cases with chromosome 17 polysomy.

The 98 cases were divided into two groups according to the expression level of the gene: a) positive CISH – *HER2/neu* gene overexpression –; b) negative CISH – absence of *HER2/neu* gene overexpression. After exposure of the data, it was performed the comparison and correlation of *SP3* clone and CISH results.

IHC methodology

The methodology for samples preparation was performed according to the manufacturer's instruction manual[19]. After preparing the slides with the selected sections, they were taken to the oven for 30 minutes at 70ºC. Antigen retrieval was performed on the PT link (Dako®) instrument for 20 minutes at 97ºC. The slides were washed for 5 minutes in wash buffer (Wash Buffer 20× concentrate). Endogenous peroxidase was inactivated with Reagent Peroxidase Blocking (Dako®) for 5 minutes, adding 100 µl over the sections. Then the solution was withdrawn from the slides, proceeding again with wash buffer. Following, 100 µl of the antibody used (*SP3* – dilution 1:500) was pipetted, maintained for 20 minutes at room temperature. The antibody was removed of the slides; and another washing was performed with wash buffer. Then the solution of horseradish peroxidase (HRP) was pipetted and left for 20 minutes at room temperature; then washed for 5 minutes in wash buffer. To the revelation, a drop of Substrate Buffer + diaminobenzidine (DAB) Chromogen on the section was added for 5 minutes. Then it was washed with wash buffer. The counterstaining was performed with hematoxylin for 10 minutes and washed twice under running water and then kept in *Scott* solution for 1 more minute. Finally, a final wash under running water was performed, followed by absolute ethanol for 30 seconds (twice) and xylene for also 30 seconds (twice).

TABLE 1 – Report of the HER2 immunohistochemical results

IHC score	Criteria	%C	%CWAF/C
0 (negative)	No immunoreactivity or immunoreactivity in less than 10% tumor cells	~60%	0%-3%
1+ (negative)	Weak immunoreactivity in more than 10% of tumor cells, but only part of the membrane is positive	~10%	0%-7%
2+ (equivocal)#	Weak to moderate complete membrane immunoreactivity in more than 10% of the tumor cells or intense circumferential membrane staining in less than 30% cells	~5%-10%	25%-35%
3+ (positive)	More than 30% of the tumor cells should show intense and uniform staining of the circumferential membrane. A homogeneous pattern must be present	~15%-20%	95%##

Adapted from: College of American Pathologists (CAP), 2012.

%C: percentage of cases; %CWAF/C: percentage of cases with amplification by FISH or CISH; #: The panel of expert recommended to test the doubtful cases to gene amplification by FISH; ##: The expert panel increased the fraction of cells with membrane staining from 10% to 30%, to increase the correlation with gene amplification by FISH. According to the panel, cancer with 3+ by IHC result should show gene amplification in at least 95% of cases.

IHC: immunohistochemistry; FISH: fluorescence in situ *hybridization; CISH: chromogenic* in situ *hybridization.*

TABLE 2 – Report of the results of study with FISH and CISH for HER2

ISH result	Critera	Comments
Positive for amplification	> 6 copies of the gene or > 2.2 ratio	> 6 copies of the gene may be present in a ratio < 2.2, if the polysomy is present. Besides the relation, the number of genes and number of 17 chromosome must be provided when they are determined as part of the test
Equivocal for amplification	4 to 6 gene or 1.8 to 2.2 ratio#	The guidelines suggest counting additional cells by FISH, retest, or performing IHC
Negative for amplification	< 4 gene or < 1.8 ratio	

Adapted from: College of American Pathologists *(*CAP*), 2012.*

ISH: in situ *hybridization; FISH: fluorescence* in situ *hybridization; CISH: chromogenic* in situ *hybridization; IHC: immunohistochemistry #: patients with ratio 2.0 or higher were eligible for Herceptin® test.*

CISH methodology

The methodology for samples preparation was performed according to the manufacturer's instruction manual[25].

Pretreatment (first day)

The sections were made at 3 µm thick and laid to dry in an oven at 70ºC for 1 hour. Following they were incubated for 10 minutes in xylene at 10 minutes at 70ºC. Then slides were incubated for 5 minutes into xylene at room temperature (twice), followed by 3 minutes in 100% ethanol (three times) and for 5 minutes in 3% hydrogen peroxide solution. They were rinsed in distilled water for 1 minute (twice), and then incubated at 95ºC in ethylenediaminetetraacetic acid (EDTA) solution for 15 minutes. Subsequently they were transferred to distilled water for 2 minutes (twice). The pepsin solution was applied and incubated for 5 minutes in a moist chamber at room temperature. After this period of time, washing the slides in distilled water was conducted. Dehydration was performed with 70%, 90% and 100% ethanol for 1 minute each; the slides were air dried.

Denaturing and hybridization

The probe was homogenized by vortexing. Pipette 8 µl into the sections, which were covered with a cover slip. Preparations denaturation was performed in hybridizer for 5 minutes at 78ºC-80ºC. The slides were left overnight, hybridizing at 37ºC.

Detection (second day)

Cover slips were removed by submerging them in Wash Buffer SSC for 5 minutes at 25ºC, and then washed for 5 minutes in the same buffer at 75ºC-80ºC. Following the slides were washed in distilled water for 1 minute (twice) and again in buffer. Anti-DIG/DNP-Mix (room temperature) was applied over the sections and incubated for 15 minutes at 37ºC in a moist chamber. Washed for 1 minute in the buffer above mentioned and applied horseradish (HRP)/alkaline phosphatase (AP)-Polymer-Mix on cuts, and incubated for 15 min at 37ºC in a moist chamber. During incubation of the slides, a AP-Red Solution (adding one drop of AP-Red Solution A in a graduated test tube, adding 1 ml of AP-Red Solution B) was prepared. It is noteworthy that the prepared solution is photosensitive. The slides were washed three times more for 1 minute in Wash Buffer tris-buffered saline (TBS) (20× concentrate); AP-Red Solution was applied incubating them for 10 minutes at room temperature, in the dark, between 7 and 15 minutes. During incubation a HRP-Green Solution (two drops of HRP-Green Solution A into a graduated tube, adding 1 ml of HRP-Green Solution B, mix thoroughly) was prepared. The slides were washed for 2 minutes in distilled water, counterstained for 2 minutes with Nuclear Blue Solution (room temperature), washed with running water and flowing three times of 30 seconds in 100% ethanol. Finally, they were flowed twice 30 seconds in xylene and mounted with mounting medium.

RESULTS

From the 98 cases studied, 48 showed positive CISH and 50 were negative. Assessing the agreement of IHC with positive CISH group, 77% (37 cases) showed agreements using the *SP3* clone (score 3+). In the same group, 6% (three cases) were negative (score 0; 1+) by IHC, and 16% (8 cases) showed inconclusive result (score 2+). Regarding the negative CISH group, 94% (47 cases) showed agreement with the gold standard assessment, while 6% (three cases) still remained uncertain in its results (**Table 3**). In this same group, there were no cases presenting positive results by IHC.

The sensitivity and specificity of clone *SP3* clone were also evaluated, using CISH as standard test. For this analysis, we used only the cases that showed negative (0/1+) and positive (3+) results by IHC (**Table 4**). After performing the calculations, *SP3* showed 92% sensitivity and 100% specificity. The positive predictive value (PPV) was 100% (NPV) was 94%; 96% accuracy.

TABLE 3 – Correlation of results between IHC and CISH

Score IHC SP3	CISH positive	CISH negative
0/1+	3	47
2+	8	3
3+	37	0
Total	48	50

IHC: immunohistochemistry; CISH: chromogenic in situ *hybridization*.

TABLE 4 – Viable results of IHC and CISH to calculate the sensitivity and specificity

SP3 scores	CISH positive	CISH negative	Total
SP3 3+	37	0	37
SP3 0/1+	3	47	50
Total	40	47	87

IHC: immunohistochemistry; CISH: chromogenic in situ *hybridization*.

DISCUSSION

A few years ago, the rabbit monoclonal antibodies have been increasingly popular. This is due to the fact they have higher affinity for the desired epitope, revealing a more intense positive staining with lower background staining, and therefore it has higher affinity and specificity than rat monoclonal antibodies[22, 28]. The rabbit monoclonal antibodies are directed to the extracellular domain of the HER2 receptor, whereas the same therapy with *Trastuzumab* also targets the extramembranar epitope of HER2; antibodies that detect this portion can produce results with greater clinical relevance regarding treatment response[14].

The percentage observed in the correlation of results between the techniques studied shows that the *SP3* clone has a good correlation when measured with the gold Standard. The negative

CISH group showed the best correlation, in which 94% (47/50) of the cases in agreement between CISH and *SP3*. In the same group, 6% (3/50) of cases were equivocal by *SP3* clone. There were no cases of de *SP3* score 3+, which revealed negativity for gene amplification.

Studies show high concordance between the *SP3* clone with FISH or CISH tests[30]. When compared with the rabbit polyclonal antibody *A0485*, some authors have noted a significant reduction of 2+ with *SP3*[31]. Manion *et al.*[22] reported that using the rabbit monoclonal antibody anti-*SP3* instead of *A0485* reduced about 50% the number of doubtful cases sent to the FISH test.

But the positive CISH group had a lower percentage of agreement, showing 77% (37/48) of the cases correlating results between *SP3* and CISH. We still must point out the cases which had false negative result and/or equivocal (one case 0, two cases 1+ and eight cases 2+), since the possible exclusion of these patients for proper treatment with *Trastuzumab*, once they are truly positive for *HER2/neu* gene amplification, further worse their life's expectation, which is already limited.

Studies report the variability of scores among observers. The scores 0 and 3+ have high agreement, in contrast to the scores 1+ and 2+, which are often interpreted with variations[18, 26, 32]. Among these indicative, it is clear that despite IHC well-known advantages by pathology laboratory, this has the disadvantage of being a semiquantitative[18, 26, 28]. This shows the need to accurately verify the gene amplification in samples that do not exhibit clear results.

The samples evaluated in this study derived from ambiguous results (score 2+) of *CB11* clone, when tested in the initial routine of the laboratory and then referred to the CISH technique for exact revealing the result through gene amplification, along with the *SP3* clone to corroborate this result. Unlike scores 0 and 3+ that have good definition standard, the score 2+ is doubtful, and often score 1+ is also interpreted in the same way. The score 1+ overestimation may occur by the pathologist when it does not have a well-defined tumor histological section or a distinct staining of tissue structures, thus causing the referring for the gold standard technique – CISH –, which enables the patient has the opportunity for a more reliable diagnosis when only subjected to IHC.

The research carried out to examine six different mouse and rabbit monoclonal and polyclonal antibodies performed by Nunes *et al.*[23] showed that the rabbit mono or polyclonal antibodies are more sensitive and ensure adequate treatment for patients with gene overexpression, including *SP3*. However, in our study, using CISH as a reference test, the *SP3* was 92% sensitivity and 100% specificity. Other studies also show greater *SP3* specificity in tendency, and less sensitivity, when compared with other clones, they were evaluated by FISH or CISH standard test[14, 22, 33]. These results are less sensitive due to the number of false negative cases (0/1+) by *SP3*, which were amplified by CISH.

The discrepancy results in IHC is reported in the literature, although this technique widely used and well accepted in pathology laboratories. There are several possible explanations for these differences, such as the use of different antibodies available on the market, the different antibody staining techniques, including dilution and antigen retrieval, the experience of individuals involved in the technique, the lack of internal quality control, the time it takes for the sample to be submerged in formalin, the duration of fixing and also the use of inappropriate fasteners[14, 21, 22, 24, 31, 33, 34]. The choice of an ideal clone to detect the protein overexpression of *HER2/neu* gene seems to have not yet been found and fully supported by a group of. According to Gown and Goldstein[31], the choice between either clones may be the less important when comparing the procedures related to the IHC in the pre-analytical and analytical phase in which these, in turn, are even less important than the ability of the pathologist in evaluating the sample and fitting it in the categories 0/1+, 2+ e 3+, knowing that interpreting variations between evaluators may occur[14, 22, 24, 31, 34].

CONCLUSION

The study found that the *SP3* clone shows good results compared to CISH technique/method. The sensitivity and specificity of *SP3* also reached satisfactory levels.

Realizing the possible differences between the results of IHC, even though this is an important and valuable tool in the daily routine of pathology laboratories, it is suggested a new analysis, with another clone, when the result of the first is not clearly obtained. Sending the inconclusive samples for genetic evaluation by ISH, whether chromogen or fluorescent, seems to be the most reliable way to ensure proper treatment of patients with breast cancer *HER2/neu* overexpressing.

RESUMO

Introdução: *Avanços no campo da biologia molecular têm proporcionado a diferenciação dos subtipos moleculares das neoplasias mamárias, fornecendo melhor prognóstico e ferramentas importantes para a terapêutica de pacientes com câncer de mama. Entre esses subtipos, as alterações ocorridas no gene receptor tipo 2 do fator de crescimento epidérmico humano* (HER2/neu*) amplificam o seu número de cópias e geram o aumento da proteína HER2. Estudos mostram que pacientes portadoras de câncer de mama HER2/neu amplificado tendem a ter recaída mais cedo e tempo de sobrevida menor, sendo o anticorpo monoclonal* Trastuzumab *a terapia indicada. A elegibilidade das pacientes para a terapia é feita inicialmente pela técnica de imuno-histoquímica (IHQ), que avalia o nível de expressão da proteína HER2. Após essa avaliação, os casos que apresentam diagnósticos equívocos (escore 2+) são encaminhados para uma técnica mais precisa, a hibridização cromógena* in situ *(CISH).* ***Objetivo:*** *Analisar a sensibilidade e a especificidade do anticorpo* SP3*, além de determinar o seu nível de concordância com a técnica de CISH.* ***Material e métodos:*** *Estudo retrospectivo no banco de dados de um laboratório anatomopatológico, em laudos de exames de CISH para HER2/neu.* ***Conclusão:*** *Os resultados revelaram que o clone* SP3 *apresentou 100% de especificidade e 92% de sensibilidade. A IHQ revela variabilidade em seus resultados, porém é sabido que a técnica é uma importante ferramenta na rotina diária dos laboratórios, contribuindo na triagem inicial das pacientes portadoras de câncer de mama, que, posteriormente, mostram resultados satisfatórios quando comparados com a técnica de CISH.*

Unitermos: *neoplasias da mama; hibridização* in situ*; imuno-histoquímica.*

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