SUMMARY

BACKGROUND. Novel rabbit monoclonal antibodies (RabMab) for estrogen (ER), progesterone (PR) receptors and HER2 evaluation by immunohistochemistry have recently been commercially released. We compared the RabMab anti-ER, anti-PR and anti-HER2 to mouse monoclonal antibodies (Mab) using tissue microarrays (TMA) of breast carcinomas.

METHODS. Two TMA containing breast carcinomas were built. Sections were immunostained using anti-ER and anti-PR, Mab and RabMab. The sections stained for ER and PR were evaluated considering positive those tumors in which more than 1% of the tumor cell nuclei stained moderate or strong. For HER2, the immunostained sections were evaluated using the ASCO/CAP guidelines for HER2. Chromogenic in situ hybridization (CISH) was used as the gold standard for HER2 evaluation. CISH was evaluated using the Zymed HER2 CISH interpretation guidelines.

RESULTS. RabMab against ER have similar staining patterns compared to the 6F11 (Mab), but stronger than 1D5 (Mab) from three different suppliers. The RabMab against PR provide stronger and sharper immunohistochemical signals compared to Mab. The detection of HER2 protein overexpression was more prevalent with the polyclonal antibodies and RabMab than with the Mab. These were more specific than the RabMab, which were more sensitive when compared to CISH.

CONCLUSION. The novel RabMab against ER and PR showed higher intensity of staining than the Mab. The RabMab against HER2 is more sensitive than Mab, however, Mab presented more specificity than RabMab when compared to CISH for HER2 evaluation of breast carcinomas.

KEY WORDS: Monoclonal antibodies. Immunohistochemistry. Breast neoplasms, hormone receptors. HER2

INTRODUCTION

The selection of patients for adjuvant therapy against breast cancer requires predictive factor evaluation for the therapeutic response. These factors include estrogen (ER) and progesterone (PR) receptors and HER21-3.

After improvement of mouse monoclonal antibodies for formalin-fixed, paraffin-embedded tissues, evaluation of hormone receptors has been made almost exclusively by immunohistochemistry (IHC). Recently, novel rabbit monoclonal antibodies were released needing comparative research with the mouse monoclonal antibodies most used4,5.

Differently from hormone receptors, HER2 evaluation in breast cancer may be carried out in two different ways: protein overexpression evaluation by IHC and gene amplification evaluation by fluorescent in situ hybridization (FISH) or chromogenic in situ hybridization (CISH). IHC is considered a scanning test for HER2 and the indeterminate cases (2+) must be confirmed by FISH or CISH techniques, which are considered gold standard6.

In this study we compared the sensitivity of novel rabbit monoclonal antibodies against ER and PR to other monoclonal antibodies most frequently used. We also compared novel rabbit monoclonal antibody SP3 and other monoclonal and polyclonal antibodies against HER2, with the chromogenic in situ hybridization (CISH) to evaluate specificity and sensitivity of the antibodies.

METHODS

Case selection

We built different TMA of breast carcinomas: one for hormone receptor analysis and the other for HER2 testing6. The
first TMA contained twenty-four cases of invasive breast carcinomas, diagnosed between 1990 and 2005, randomly selected for ER and PR evaluation from the files of the Breast Pathology Laboratory of the School of Medicine, Federal University of Minas Gerais, Brazil. All original slides were reviewed to confirm diagnosis and select representative areas of tumors. Two cylinders (2mm diameter) of each tumor with representative areas of neoplasia were selected from paraffin blocks to build the TMA.

The second TMA was built using paraffin embedded tissue samples from 84 breast invasive and intraductal carcinomas examined between 1987 and 2005, selected for HER2 evaluation. These cases were selected based upon existing results of HER2 tests for clinical management using CB11 antibody and blocks suitable for TMA construction. To amplify our casuistry of HER2 overexpressing tumors for comparison of different antibodies, we selected 45 previously tested cases scored as 2+ or 3+, and 39 previously tested cases scored as 0 or 1+. Hematoxylin and eosin (H&E) stained slides of the corresponding samples were reviewed and tumors were classified and graded based on criteria of the College of American Pathologists (1999) and Elston & Ellis (1991)\textsuperscript{8, 9}. Two representative areas of each tumor were identified and marked on H&E stained slides and the corresponding paraffin embedded tissue blocks were obtained for the construction of the TMA block.

Cylinders of tumors previously tested for ER, PR and HER2 whose results were positive, were also included in both TMA to be used as internal controls. Sequential 5μm sections were obtained from the TMA and stained for haematoxylin and eosin (H&E) to confirm tumor diagnosis. The interval sections were used for immunohistochemical and CISH study in HER2 evaluation. Slides containing sections of positive breast tumors for ER, PR and HER2 were also included in all batches as an external control for all markers.

**Immunohistochemical and CISH procedures**

The sections were mounted on glass slides coated with silane (3-aminopropyltrithoxysilane) and dried for 30 minutes at 37°C. Sections were deparaffinized in xylene and rehydrated via a series of graded alcohols. Endogenous peroxidase activity was blocked by incubating the sections in a methanol bath containing 3% hydrogen peroxide for 20 min, followed by washing in distilled water. Antibodies, dilutions and antigen retrieval methods used for ER, PR, and HER2 evaluation are summarized in Table 1. The HercepTest was used following the instructions provided by the kit. After the antigen retrieval method, the primary antibody was applied and incubated for 90 minutes at room temperature. Preliminary testing was performed in our laboratory to identify the best concentration for each antibody and choose the negative and positive controls using the dilution data supplied by the manufacturer, as the starting point. After washing the primary antibody with phosphate buffered saline (PBS), slides were incubated with linking biotinylated antibody (Super Sensitive Link, Label IHC Detection System, BiogenexT) for 20 min. The sections were rinsed with PBS, followed by incubation with peroxidase-conjugated streptavidin complex for 20 min (Super Sensitive Link, Label IHC Detection System, and BiogenexT). Freshly prepared DAB solution (1 drop of 3,3'-diaminobenzidine tetrahydrochloride for 1ml of substrate, DAKOT) was applied on each section for 2 minutes. DAB was removed by rinsing with distilled water. The slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted using entellan.

**CISH was performed at The Breakthrough Breast Cancer Research Centre, London; England (J.S.R.-F., M.B.L.). The**

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### Table 1 - Clones, antigen, types, sources, dilutions and antigen retrieval methods used in the immunohistochemical reactions

<table>
<thead>
<tr>
<th>Clone</th>
<th>Type of antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Antigen retrieval/time</th>
</tr>
</thead>
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<tr>
<td>Anti-ER</td>
<td>SP1</td>
<td>Rabbit Monoclonal</td>
<td>LabVision, USA</td>
<td>1:300</td>
</tr>
<tr>
<td></td>
<td>B644</td>
<td>Rabbit Monoclonal</td>
<td>CellMarque, USA</td>
<td>1:300</td>
</tr>
<tr>
<td></td>
<td>1D5</td>
<td>Mouse Monoclonal</td>
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<td>1:100</td>
</tr>
<tr>
<td></td>
<td>1D5</td>
<td>Mouse Monoclonal</td>
<td>CellMarque, USA</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>1D5</td>
<td>Mouse Monoclonal</td>
<td>Biogenex, USA</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>6F11</td>
<td>Mouse Monoclonal</td>
<td>Leica, UK</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-PR</td>
<td>SP2</td>
<td>Rabbit Monoclonal</td>
<td>LabVision, USA</td>
<td>1:300</td>
</tr>
<tr>
<td></td>
<td>B645</td>
<td>Rabbit Monoclonal</td>
<td>CellMarque, USA</td>
<td>1:300</td>
</tr>
<tr>
<td></td>
<td>Pg312</td>
<td>Mouse Monoclonal</td>
<td>Leica, UK</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Pg636</td>
<td>Mouse Monoclonal</td>
<td>Dako, USA</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-HER2</td>
<td>SP3</td>
<td>Rabbit Monoclonal</td>
<td>LabVision, USA</td>
<td>1:300</td>
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<tr>
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<td>Rabbit Polyclonal</td>
<td>Dako, USA</td>
<td>Prediluted</td>
</tr>
<tr>
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<td>A0485</td>
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<td>Dako, USA</td>
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<tr>
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<td>Mouse Monoclonal</td>
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<tr>
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<td>CM-CB11</td>
<td>Mouse Monoclonal</td>
<td>CellMarque, USA</td>
<td>Prediluted</td>
</tr>
<tr>
<td></td>
<td>4D5</td>
<td>Mouse Monoclonal</td>
<td>Genentech, USA</td>
<td>1:250</td>
</tr>
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</table>
Zymed HER2 Spot-Light kit (Zymed, South San Francisco, USA) was used according to the manufacturer’s instructions.

**Immunostaining analysis**

All sections submitted to immunohistochemistry were coded. For ER and PR evaluation, the same examiner (RMR) reviewed all slides without knowledge of the antibody used. The Allred’s scoring system recommended by the St. Gallen Conference, 2007 was used. We considered positive those tumors containing more than 1% of stained nuclei, staining moderate or strong. The strongest staining hot spot of the two discs of each tumor was considered in the analysis. The background was also evaluated and scored as negative (0), weak (1), moderate (2), and strong (3).

For HER2, the immunostained sections were evaluated by the same examiner (CBN) blinded from the results of CISH analysis based on the new ASCO/CAP Guideline Recommendations for HER2 testing (0, no staining or membrane staining is observed in less than 10% of tumor cells; 1+, faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells, and only part of the membrane is stained; 2+, weak to moderate complete membrane staining is observed in more than 10% of the tumor cells; and 3+, strong complete membrane staining is observed in more than 30% of the tumor cells). Cases interpreted as 0 or 1+ were considered negative, those interpreted as 2+ were considered borderline (indeterminate or equivocal) and 3+ were considered positive. Cytoplasmic immunostaining was not incorporated into the final scoring. All doubts and 10% of all sections stained for ER, PR and HER2 were reviewed by an experienced breast pathologist (HG) using a double head microscope.

**CISH analysis**

CISH was evaluated using the Zymed HER2 CISH interpretation guidelines. At least 30, non-equivocal and non-overlapping neoplastic cells were counted per case. Nonamplified cases were defined as those with one to five signals per nucleus in >50% of tumor cells; amplification was defined as i) more than 5 gene copies per nucleus in >50% of tumor cells, ii) when small or large gene copy clusters were found in >50% of tumor cells.

**Statistical analysis**

The Software SPSS, Version 12.0, was used for statistical analysis. Wilcoxon test was used to compare the different groups of categorical variables. The Spearman Coefficient was used to evaluate the positive correlation among the categorical variables of the different groups. The Kappa test was applied to compare the RabMab SP3 Her2 overexpression with the other antibodies. CISH test was used as the “gold standard”, and overall sensitivity and specificity were calculated for each antibody staining.

**Results**

**Estrogen receptor analysis**

The estrogen receptor was positive in 15 out of 24 tumors (62.5%), with variable staining intensity among the different cases and antibodies used. In one case, there was no variation in the intensity of staining using the different clones and tumor cells showed strong reactivity. In five cases, there were positive reactions for some antibodies (including both rabbit antibodies) and negative reactions for the other antibodies. In all five cases, the reaction was negative for 1D5 (Biogenex). Two cases were negative for 1D5 from three suppliers and positive for both rabbit and mouse antibodies 6F11. Nine cases (37.5%) were negative for all antibodies tested.

There was a statistically significant difference when comparing the clone 1D5 from different suppliers to both rabbit antibodies, which stained stronger (p<0.05). However, no difference was observed between the stains of both the rabbit and mouse antibody 6F11. A statistically significant difference was found when comparing all antibodies to the clone 1D5 (Biogenex), which showed weaker reactions than the other antibodies (p<0.02).

The highest correlation was obtained with the two rabbit clones (SP1 and B644) and the mouse clone 6F11, which showed stronger nuclear staining when compared to the other mouse antibodies, in the majority of cases.

**Progesterone receptor analysis**

Sixteen out of 24 tumors (66.7%) were positive for PR. In 8/16 cases, there was no variation in the intensity of the staining using different clones. In four cases, the reactions were positive for rabbit (SP2 and B645) but negative for mouse (Pgr312 and Pgr636) antibodies. One case was positive for both rabbit and negative for both mouse antibodies. Eight cases (33.4%) were negative for all antibodies tested. Two cases were negative for ER and positive for PR, and one case was negative for PR and positive for ER. Sections that stained for both SP2 and B645 showed significantly stronger staining intensity than those stained for both PR mouse antibodies (p=0.025).

There was a statistically significant agreement between the estrogen and progesterone receptor positivity and negativity in the same cases (p=0.001). The negative control case included in the study was confirmed to be negative with all ER and PR antibodies tested.

**HER2 analysis**

The detection of HER2 protein overexpression was more prevalent with the polyclonal antibodies A0485 (56 cases, 66.7%), HercepTest (46 cases, 54.8%) and the RabMab SP3 (48 cases, 57.1%) than with the monoclonal antibodies CB11 (38 and 39 cases, 45.2%) and 4D5 (40 cases, 47.6%). There was a 96.7% concordance between 2+ and 3+ results obtained with SP3 and A0485 antibodies. The concordance between 2+ and 3+ results obtained with SP3 and HercepTest, SP3 and 4D5, and SP3 and CB11 antibodies was 83.6%, 77% and 73.8% respectively (Table 2). The concordance (unweighted Kappa scores) between SP3 and HercepTest, A0485, 4D5 and both CB11 was 0.74, 0.71, 0.65 and 0.61, respectively.

**CISH analysis**

CISH identified HER2 gene amplification in 46 tumors (54.8%). All rabbit monoclonal and polyclonal antibodies (SP3,
A0485, and HercepTest) presented 100% sensitivity and specificity ranging from 64.3 (A0485) to 94.1 (HercepTest). Two (5.2%) HercepTest, 5 (13.1%) SP3 and 10 (26.3%) A0485 nonamplified cases were scored as 3+. Mouse monoclonal antibodies presented a sensitivity ranging from 92.5 (CB11) to 97.4 (4D5), and a specificity ranging from 94.7 (4D5) to 97.3 (CB11) when compared to CISH results; 3/46 (6.5%) of HER2 CISH amplified cases did not show CB11 immunoreactivity. In summary, SP3, A0485 and HercepTest allowed a higher working dilution. Cheang et al.14 published a study of rabbit monoclonal antibodies against estrogen and progesterone receptors in fine-needle aspirates and paraffin-embedded sections from breast cancers using SP1 and SP2 rabbit antibodies. They found that use of rabbit monoclonal antibodies against ER and PR on alcohol-fixed smears and paraffin sections provided several advantages such as high sensitivity and specificity of the reaction, stronger immunostaining, shorter procedure times, and avoidance of the antigen retrieval step. Rossi et al.13 carried out a comparative study of rabbit monoclonal antibodies against estrogen and progesterone receptors and other markers, and of mouse monoclonal antibodies against the same antigens on several tumor types. They found no significant differences in the percentage of positive cells and staining intensity. However, these authors suggest that rabbit antibodies appear to offer increased sensitivity with no apparent loss of specificity and also allowed a higher working dilution. Cheang et al.14 published a robust study that evaluated immunohistochemistry using the new rabbit antibody SP1 and the mouse antibody 1D5. They evaluated the relationship to biochemical ER assay results and clinical data on survival and adjuvant systemic therapy. These authors14 considered SP1 as an improved standard for ER immunohistochemistry assessment in breast cancer when compared to 1D5. The information of sensitivity and specificity of SP1 provided by these authors encouraged us to carry out the present study including clones 1D5 from different suppliers and clone 6F11 against ER, and also comparing mouse and rabbit monoclonal antibodies against PR. The purpose of our paper was neither to correlate patient therapeutic response nor to establish sensitivity, specificity, and a gold standard for ER and PR, using the current methodology.

Scores obtained in our study using SP1, B644 and 6F11, when compared to those of other antibodies led us to distinguish the clones SP1, B644 and 6F11 as the antibodies that showed the highest staining intensity against ER (SP1 and 6F11) and PR (B644). These findings suggest that the rabbit antibodies and the 6F11 can be used at higher working dilutions when compared to mouse monoclonal antibodies.

According to Huang et al.5, the high affinity of the clone SP1 and its binding to a different epitope from clone 1D5 would explain why antigen retrieval is not necessary. The rabbit SP1 has appropriate tissue reactivity, with nuclear staining in epithelial tissues of known ER status showing an affinity 8 times higher than that of 1D5 and reactivity with the predicted band on Western blots.6

There are few studies comparing different mouse clones against PR and only one study comparing rabbit and mouse clones anti-PR which compared the rabbit anti-PR SP2 to the mouse 1A64. The authors showed that the antibody affinity of SP2 is 12 times higher than that of 1A64. In our study, we found a significantly better staining for rabbit clones SP2 and B645 as compared to the mouse clones PgR636 and PgR312.

CISH has been validated in many reports compared to FISH, with a high concordance rate12-18. The current HER2 CISH is based on single-color detection, without centromere 17 correction. Despite the fact that some authors consider the chromosome 17 copy correction to be essential19, 20, there is no international consensus about which system should be used. For clinical assessment of HER2 status, aneusomy 17 was not a significant factor for IHC-FISH discordance in most cases21, and patients with polysomy 17 tumors can respond to trastuzumab monotherapy22. In a recent trial for docetaxel or vinorelbine with or without trastuzumab, single probe CISH was successfully used as part of the entry criteria23.

Our study included HER2 previously tested and selected tumors, most of them diagnosed as ductal high grade invasive carcinoma, and HER2 overexpression ranged from 45.2% (CB11) to 66.7% (A0485). When SP3 was compared with other antibodies, the highest concordance rate was with HercepTest followed by A0485, both polyclonal antibodies. When we compared IHC with CISH results, SP3 displayed an optimal sensitivity, similar to that obtained with anti-HER2 polyclonal antibodies (i.e. HercepTest and A0485). However, 2 (5.2%) HercepTest, 5 (13.1%) SP3 and 10 (26.3%) A0485 nonamplified cases were scored as 3+. Based on the concept of oncogene addiction and the requirement of genomic changes, there is a growing belief that only HER2 amplified cases respond...
to trastuzumab. Hence it is yet to be determined if these patients would actually benefit from trastuzumab or HER2 small molecule tyrosine kinase inhibitors.

Although less sensitive, both mouse anti-Her2 antibodies (CB11 and 4D5) showed more specificity than the rabbit monoclonal SP3. Mab membrane staining favors the presence of gene amplification; however, we observed 3/46 (6.5\%) of HER2 CISH amplified cases with no CB11 immunoreactivity. These patients would not receive the appropriate treatment and, therefore, these antibodies should be used with caution.

Based on our study, we have two categories of antibodies. First, the most sensitive ones (i.e. HercepTest, SP3 and A0485), which guarantee that patients with gene-amplified tumors will receive the most appropriate treatment. However, patients with nonamplified tumors would receive a costly and cardiototoxic treatment. And second, the most specific antibodies (i.e. CB11, 4D5) that have a remarkably low prevalence of false positives, however owing to their suboptimal sensitivity, up to 6.5\% of the patients with amplified-gene tumors would be denied anti-HER2 therapy. In the light of these findings the doubt that still remains would be the choice of the ideal antibody for the detection of HER2 protein overexpression.

**Conclusion**

The new rabbit monoclonal antibodies against ER (SP1 and B644) and PR (SP2 and B645) provide stronger staining intensity than the mouse monoclonal antibodies for immunohistochemical evaluation in breast carcinomas. Our results demonstrate that rabbit antibodies against ER have similar staining patterns compared to mouse 6F11 but better than clone 1D5 from three distinct suppliers while using the same immunohistochemical protocol. The rabbit antibodies against PR (SP2 and B645) provide a stronger and sharper immunohistochemical signal compared to mouse antibodies (PgR636 and PgR312). Further studies are necessary to confirm if high sensitivity correlates to therapeutic response. The rabbit monoclonal SP3 is more sensitive than mouse monoclonal antibodies, staining similar to HercepTest for HER2 assessment. CB11 and 4D5 show higher specificity than SP3.

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**Interest conflict:** none

**RESUMO**

**Anticorpos de coelho para avaliação de receptores hormonais e HER2 em câncer de mama**

**OBJETIVOS.** Novos anticorpos monoclonais de coelho (RabMab) para a avaliação imuno-histoquímica de receptores de estrógeno (RE), progesterona (RP) e HER2 foram lançados comercialmente. Comparamos os RabMab anti-RE, anti-RP e anti-HER2 com os anticorpos monoclonais de camundongo (Mab) utilizando tissu microarrays (TMA) de carcinomas de mama.

**MÉTODOS.** Foram construídos dois TMAs de carcinomas de mama. As secções foram marcadas usando anti-RE, anti-RP e anti-HER2, Mab e RabMab através de imuno-histoquímica. As secções marcadas para RE e RP foram avaliadas considerando positivos aqueles tumores nos quais mais de 1\% dos núcleos coraram moderadamente ou forte. Para HER2, as secções foram avaliadas utilizando as recomendações da ASCO/CAP para HER2. Hidratação in situ cromogênea (CISH) foi usada como padrão-ouro para avaliação de HER2. CISH foi avaliado utilizando as recomendações da Zymed.

**RESULTADOS.** Os RabMab anti-RE apresentam intensidade de coloração semelhante ao 6F11 (Mab), porém maior que o 1D5 (Mab) proveniente de três diferentes fabricantes. Os RabMab anti-RP apresentaram sinal imuno-histoquímico mais forte e delimitado comparado aos Mab. A detecção da superexpressão da proteína HER2 foi mais prevalente entre os anticorpos policlonais e RabMab, que se mostraram mais sensíveis quando comparados com o CISH.

**CONCLUSÃO.** Os novos RabMab anti-RE e RP proporcionaram maior intensidade de coloração e os Mab. O RabMab anti-HER2 apresentou maior sensibilidade e os Mab, porém os Mab apresentaram maior especificidade quando comparados com o CISH para a avaliação de HER2 em carcinomas de mama. [Rev Assoc Med Bras 2009; 55(2): 163-8]


**References**