A comparison between the novel rabbit monoclonal antibodies (SP1 and B644) and mouse antibodies for evaluating estrogen receptor in breast tumors

Primeira submissão em **23/03/07**
Última submissão em **01/08/07**
Aceito para publicação em **13/08/07**
Publicado em **20/12/07**

Uma comparação entre os novos anticorpos monoclonais de coelho (SP1 e B644) e anticorpos de camundongo para detecção de receptores de estrógeno em carcinomas mamários

Rafael Malagoli Rocha[1]; Cristiana Buzelin Nunes[2]; Gislene Fátima Silva Rocha[3]; Flávio Nepomuceno Oliveira[4]; Fernanda Squárcio Fernandes Sanches[5]; Helenice Gobbi[6]

key words

Monoclonal antibodies

Immunohistochemistry

Breast cancer

abstract

Background: A novel generation of rabbit monoclonal antibodies has been released recently for estrogen (ER) and progesterone (PR) receptor evaluation in breast cancer by immunohistochemistry. Aims: We compared novel rabbit monoclonal antibodies anti-ER SP1 (LabVision®) and B644 (Cell Marque®) to mouse monoclonal antibodies 1D5 (Dako®) and 6F11 (Novocastra®) using a tissue microarray of breast carcinomas. Methods: Two cylinders (2 mm diameter) of formalin-fixed paraffin embedded tissue were obtained from 24 invasive breast carcinomas and immunostained by using the anti-ER rabbit and mouse antibodies and the streptavidin-biotin detection system (Biogenex®). Immunostaining was evaluated considering positive those tumors in which more than 10% of the tumor cell nuclei stained. The stain intensity was also evaluated as weak (1), moderate (2), and strong (3). Results: Both rabbit antibodies against ER have similar staining pattern to each other and also to 6F11, but significantly stronger scores compared to mouse 1D5. The rabbit antibodies allow better cost/benefit because of higher working dilutions compared to mouse antibodies using the same procedure. Conclusion: The new rabbit antibodies against ER are highly sensitive and reliable in clinical and research immunohistochemical testing of breast carcinomas.

unitermos

Anticorpos monoclonais

Imuno-histoquímica

Câncer de mama

resumo

Introdução: Uma nova geração de anticorpos monoclonais de coelho tem sido produzida para detecção de receptores de estrógeno (RE) e progesterona (RP) pela imuno-histoquímica em câncer de mama. Objetivo: Comparamos os novos anticorpos monoclonais de coelho anti-RE SP1 (LabVision®) e B644 (Cell Marque®) com anticorpos monoclonais de camundongo 1D5 (DAKO®) e 6F11 (Novocastra®) utilizando um tissue microarray de carcinomas mamários. Metodologia: Dois cilindros (2 mm de diâmetro) de tecido fixado em formol e embebido em parafina foram retirados de 24 carcinomas mamários invasivos e corados pela imuno-histoquímica utilizando-se os anticorpos de coelho e de camundongo anti-RE e o sistema de detecção estreptavidina-biotina peroxidase (Biogenex®). A coloração imuno-histoquímica foi avaliada considerando positivos os tumores nos quais mais de 10% dos núcleos das células tumorais estivessem corados. A coloração também foi classificada em fraca (1), moderada (2) e forte (3). Resultados: Ambos os anticorpos monoclonais de coelho contra RE apresentaram intensidade de coloração semelhante àquela pelo anticorpo de camundongo 6F11, porém os anticorpos de coelho apresentaram intensidades de coloração significativamente mais fortes que as do clone de camundongo 1D5. As altas diluições possíveis utilizando anticorpos de coelho permitem melhor custo/benefício quando comparadas com as diluições possíveis utilizando anticorpos de camundongo. Conclusão: Os novos anticorpos monoclonais de coelho anti-RE são altamente sensíveis e fidedignos para testes imuno-histoquímicos tanto para a clínica quanto para pesquisa de tumores mamários.

1. Doctorate student from the post-graduation program in pathology, Universidade Federal de Minas Gerais (UFMG).
2. Master's degree student from the post-graduation program in pathology, UFMG.
3. Scientific initiation scholar from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).
4. Technical support scholar from CNPq.
5. Scientific initiation scholar from Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).
6. Doctor; assistant professor at the Department of Pathological Anatomy, School of Medicine, UFMG.

This work was conducted at the breast pathology laboratory of the School of Medicine, UFMG, with financial support of FAPEMIG and CNPq. It was presented at the 95th United States and Canadian Academy of Pathology Meeting, March 2006.

Introduction

Estrogen receptor (ER) may be the best example of a tumor biomarker with an assay that drives therapeutic decision-making[7, 17]. Since 1990, pathology has played an important role not only in diagnosis, but also by providing additional information about prognostic and predictive molecular markers aimed at the best breast cancer treatment[22].

Although predictive factors of therapy response have more clinical value, the only broadly validated predictive factors for routine clinical use are ER and progesterone receptor (PR)[2]. Since the release of monoclonal and polyclonal antibodies that react in formalin-fixed paraffin-embedded tissue, scientists have been evaluating ER almost exclusively by immunohistochemistry[1, 5, 8, 19, 25]. Several different methods and different antibodies against ER have been used for immunohistochemistry testing. As a result, there is not yet a universally accepted standard[16, 23]. As with any laboratory method, technical details have a great effect on the final result[11, 20, 21].

The most used antibodies for ER evaluation by immunohistochemistry have been the mouse monoclonal antibodies. Recently, a new generation of rabbit monoclonal antibodies has been developed[14]. The technology to prepare these antibodies from a single hybridoma allows the production of antibodies with high sensitivity and specificity, high working dilutions and better cost versus benefits. According to the suppliers, the novel rabbit monoclonal antibodies could replace the mouse monoclonal antibodies because of their high specificity, lower cost, and faster technical procedure, which would not require an antigen retrieval step[3, 14, 24].

Several anti-ER antibodies have been used in clinical and research immunohistochemical testing, including mouse monoclonal antibodies. Clones 6F11 and 1D5 have been compared in clinical studies and were shown to have similar sensitivities[28]. Studies have also compared 1D5 with a novel rabbit monoclonal, SP1[15, 24], which has eight-fold higher affinity[15]. Another study showed that SP1 is more sensitive than 1D5 for detecting ER expression in breast cancer, in both a duplicate-redundancy 431-sample TMA, and in 121 whole sections of clinical materials from multiple institutions[27].

In the present study, we compared sensitivity and cost/benefit between the novel rabbit monoclonal antibodies and two most used mouse monoclonal antibodies, developing the antigen retrieval step in all immunohistochemistry reactions for ER evaluation in invasive breast carcinomas.

Methods

Case selection

Twenty-four cases of invasive breast carcinomas diagnosed between 1990 and 2005 were randomly selected from the files of the breast pathology laboratory of the School of Medicine, Universidade Federal de Minas Gerais (UFMG), Brazil. All original slides were reviewed to confirm the diagnosis and to select representative areas of tumors. Two cylinders (2 mm diameter) of each tumor with representative areas of neoplasia were selected from paraffin blocks to build a tissue microarray (TMA). Two cylinders of tumor from previously tested positive and negative tumors were also included as internal controls for TMA. Sequential 5 µm sections were obtained and stained for hematoxylin and eosin (first and last sections) to confirm diagnosis, and interval sections were used for the immunohistochemical study. Slides containing sections of a positive breast tumor were included in all batches as external control.

Immunohistochemical study

The sections were mounted on glass slides coated with silane (3-aminopropyltriethoxysilane) and dried for 30 minutes at 37°C. Then, they 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. All sections were initially submitted to heat-induced epitope retrieval using citrate buffer (pH 6.0). After that, the primary antibody was applied and incubated for 90 minutes at room temperature. Mouse monoclonal antibodies (1D5 and 6F11) and rabbit monoclonal antibodies (SP1 and B644) were used in order to evaluate ER and PR (**Table 1**). Preliminary testing was performed in our laboratory to identify the best concentration for each antibody, and to choose the negative and positive controls using the dilution data supplied by the manufacturer as the starting point. A dilution of 1:300 was used for both rabbit antibodies and 1:100 for both mouse antibodies. After washing primary antibody with phosphate buffered saline (PBS), the slides were incubated with linking biotinylated antibody (Biogenex®) for 20 min. The sections were rinsed with PBS, followed by incubation with peroxidase-conjugated streptavidin complex for 20 min (Super Sensitive Link-Label Immunohistochemical Detection System, Biogenex®).

Freshly prepared diaminobenzidine (DAB) solution (1 drop of 3, 3'-diaminobenzidine tetrahydrochloride for 1 ml of substrate, DAKO®) was applied for two minutes on each section. DAB was removed by rinsing with distilled water. The slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted using Entelan®.

Immunostaining analysis

All slides submitted to immunohistochemistry were coded, and the examiner was blinded to the antibody used. The cut offs were those most used in literature[8-10]. We considered positive those tumors containing more than 10% of stained nuclei, independent of the staining intensity. If a difference of staining was observed, the strongest staining hot spot of the two discs of each tumor was considered in the analysis. The intensity of the reaction and the background were also evaluated as negative (0) or positive: weak (1), moderate (2), and strong (3).

Statistical analysis

The Wilcoxon test was used in the comparative statistical analysis of the different antibodies positivity and the Spearman coefficient was used to evaluate if there was a positive correlation among the different antibodies.

Table 1 Evaluation of estrogen receptor in 24 breast cancers using two rabbit monoclonal antibodies (SP1 and B644) and mouse monoclonal antibodies (1D5 and 6F11)

Cases	Rabbit		Mouse	
	SP1	B644	1D5	6F11
1	1	2	2	3
2	2	1	1	2
3	0	0	0	0
4	3	2	2	3
5	0	0	0	0
6	3	3	2	3
7	2	2	1	2
8	3	3	3	3
9	2	2	0	2
10	3	3	3	3
11	0	0	0	0
12	0	0	0	0
13	2	2	1	1
14	0	0	0	0
15	3	3	3	3
16	3	3	3	3
17	3	3	3	3
18	3	3	1	2
19	0	0	0	0
20	0	0	0	0
21	1	1	0	1
22	0	0	0	0
23	3	3	2	3
24	0	0	0	0

Intensity of staining ranging from negative (0) to positive: weak (1), moderate (2), and strong (3).

Results

After developing the immunohistochemistry staining including the antigen retrieval step, the analysis of the ER stained sections generated scores that are shown in **Tables** 1 and **2**, and in **Figure 1**. Estrogen receptor evaluation was positive in 15 cases (62.5%), with variable staining intensity among the different cases and antibodies used. In five cases (8, 10, 15, 16, and 17), there was no variation in the intensity of staining using the different clones, and tumor cells showed strong reactivity. In two cases (9 and 21) there was positive reaction for both rabbit antibodies and mouse 6F11 and negative reaction for mouse 1D5. Nine cases (37.5%) were negative for all antibodies tested.

There was a statistically significant difference when comparing clone 1D5 to both rabbit antibodies and to mouse 6F11, which stained stronger ($p < 0.05$). However, no difference was observed between the stains of both rabbits and mouse 6F11. Besides, they showed the highest agreement (Figure 1).

Time of storage of the paraffin blocks, which ranged from one to 15 years, had no effect on staining intensity and positivity.

The costs of each test (in US$) of all antibodies are shown on **Table 3**.

Discussion

In the present study we compared the immunohistochemistry staining of the novel rabbit monoclonal antibodies SP1 and B644 with mouse monoclonal antibodies 1D5 and 6F11 for ER testing in breast carcinomas using TMA and the same immunohistochemical method and antigen retrieval. There are few other studies in literature comparing the novel rabbit monoclonal antibodies to mouse antibodies[3, 4, 24] and no studies comparing both rabbit antibodies. Cano *et al.*[3] evaluated ER and PR in fine-needle aspirates and paraffin-embedded sections from breast cancers using SP1 and SP2 rabbit antibodies. They

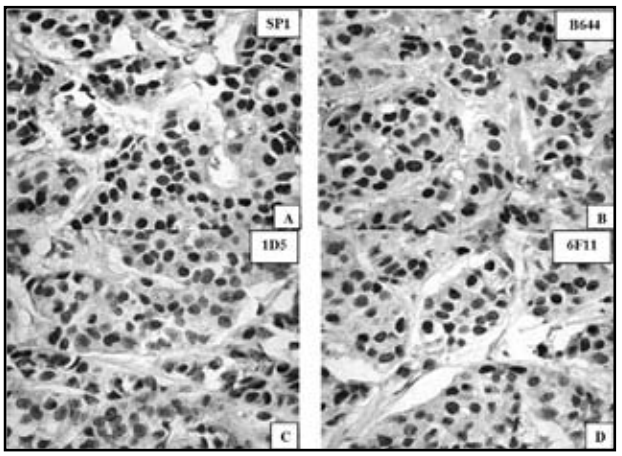

Figure 1 – *Estrogen receptor: sections stained for both rabbit antibody SP1 (A) and B644 (B) showing similar stain intensity to sections stained for mouse 6F11 (D) and stronger staining intensity compared to mouse 1D5 (C) observed at high magnification*

Table 2 Number of positive and negative cases and percentages (in parenthesis) according to staining intensity for each antibody tested against estrogen receptor

Staining intensity	Rabbit		Mouse	
	SP1	B644	1D5	6F11
0	9 (37,5)	9 (37,5)	11 (45,8)	9 (37,5)
1	2 (8,4)	2 (8,4)	4 (16,7)	2 (8,4)
2	4 (16,7)	5 (20,8)	4 (16,7)	4 (16,7)
3	9 (37,4)	8 (33,3)	5 (20,8)	9 (37,4)
Total	24 (100)	24 (100)	24 (100)	24 (100)

Intensity of staining ranged from negative (0) to positive: weak (1), moderate (2), and strong (3).

Table 3 Prices per test of each monoclonal

Antibody type	Clone	Supplier	Price per test (US$)
Rabbit anti-ER	SP1	Lab Vision	0.46
	B644	Cell Marque	0.33
Mouse anti-ER	1D5	DAKO	1.56
	6F11	Novocastra	0.98

found that the 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 antigen retrieval step. Rossi *et al.*[24] carried out a comparative study between rabbit clones against estrogen and progesterone receptors, Ki67, cyclin D1, CD3, CD5, CD23, and synaptophysin and classic mouse monoclonal antibodies against the same antigens on several tumor types as well as normal tissues. They found no significant differences in the percentage of positive cells and staining intensity. However, the authors suggest that the rabbit antibodies appear to offer increased sensitivity with no apparent loss of specificity and also allowed a higher working dilution. Cheuk *et al.*[6] compared the novel rabbit anticyclin D1 to the mouse antibodies against cyclinD1 and considered that the rabbit antibody SP4 showed superior performance over the mouse monoclonal antibody. A consistent immunostaining for cyclin D1 was readily achieved when compared to mouse antibody DCS-6 in 150 cases of lymphoproliferative lesions[9]. Cheang *et al.*[4] evaluated immunohistochemistry using the new rabbit antibody SP1 and mouse antibody 1D5. They evaluated the relationship to biochemical ER assay results and clinical data on survival and adjuvant systemic therapy. The authors detected 69.5% of positivity when using the rabbit SP1 and 63.1% using the mouse antibody 1D5. Rabbit antibody SP1 was also a better independent prognostic factor than 1D5 in multivariate analysis, including age, tumor size, grade, and lymphovascular and nodal status. SP1 was considered, by these authors[4], an improved standard for ER immunohistochemistry assessment in breast cancer. In our study, we considered the cut point of 10% for positivity. However, there is a variation of ER scoring interpretation in the literature, ranking from 1% to 10% considering or not the staining intensity. Harvey *et al.*[13] demonstrated that patients whose tumors presented 1% or more ER-positive cells responded to antiestrogen therapy.

A semiquantitative evaluation of the intensity of staining was used in order to detect differences of sensitivity among the different antibodies. Many studies reported their results of ER and PR analysis based on a semiquantitative method considering both intensity and percentage of positive nuclei. However, other investigators excluded the intensity of staining, as it has been shown to have no relationship to therapy response or prognosis[1, 8, 12, 18, 26].

Although the suppliers of the rabbit monoclonal antibodies advocate that the total reaction time can be shorter by avoiding the epitope retrieval step, pretests performed in our laboratory showed that the working dilution was 1:50 when antigen retrieval was not used, whereas a dilution of 1:300 was achieved when using epitope retrieval. Although the antigen retrieval step is more time-consuming, the total cost of each test dropped six times when this technique was used in our slides. Other authors used rabbit monoclonal SP1 10 times less concentrated than 6F11, when using antigen retrieval. They also described that acceptable staining was obtained with SP1 even in the absence of antigen retrieval, but 6F11 sensitivity dropped dramatically[24]. In our experience, comparison among the costs of each test for each antibody showed that the rabbit monoclonal antibodies present lower cost per test due to their higher working dilutions. However, if the recommended dilution supplied by the manufacturers had been used, the cost per test would be quite similar for the different antibodies. According to Huang *et al.*[14], who did not perform antigen retrieval step, the high affinity of clone SP1 and its binding to a different epitope from clone 1D5 would explain why antigen retrieval is not necessary. Rabbit monoclonal antibody SP1 has appropriate tissue reactivity, with nuclear staining in epithelial tissues of known ER status, showing an affinity eight times higher than that of 1D5 and reactivity with the predicted band on Western blotting[15].

Huang *et al.*[15] also demonstrated that SP1 showed similar results to mouse clone 1D5, in all but six out of 61 samples. According to the authors, clone SP1 may be more sensitive than clone 1D5 and has the same specificity as clone 1D5 in immunohistochemistry[15].

In summary, the new rabbit monoclonal antibodies against ER (SP1 and B644) are highly sensitive, showing a stronger and sharper immunohistochemistry signal, and reliable in immunohistochemical testing of breast carcinomas. Both ER rabbit antibodies allowed better cost/benefit because of higher working dilutions compared to mouse 1D5 and 6F11 and are sensitive and reliable for clinical and research testing.

References

1. Anderson, J.; Poulsen, H. S. Immunohistochemical estrogen receptor determination in paraffin-embedded tissue: prediction of response to hormonal treatment in advanced breast cancer. *Cancer*, v. 64, p. 1901-89, 1908.

2. Barnes, D. M. *et al.* Immunohistochemical determination of oestrogen receptor: comparison of different methods of assessment of staining and correlation with clinical outcome of breast cancer patients. *Br J Cancer*, v. 74, p. 1445-51, 1996.

3. Cano, G. *et al*. Estimation of hormone receptor status in fine-needle aspirates and paraffin-embedded sections from breast cancer using the novel rabbit monoclonal antibodies SP1 and SP2. *Diagn Cytopathol*, v. 29, n. 4, p. 207-11, 2003.
4. Cheang, M. C. *et al*. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. *J Clin Oncol*, v. 24, n. 36, p. 5626-8, 2006.
5. Chebil, G. *et al*. Estrogen and progesterone receptor assay in paraffin-embedded breast cancer – reproducibility of assessment. *Acta Oncol*, v. 43, n. 1, p.43-7, 2003.
6. Cheuk, W. *et al*. Consistent immunostaining for cyclin D1 can be achieved on a routine basis using a newly available rabbit monoclonal antibody. *Am J Surg Pathol*, v. 28, n. 6, p. 801-7, 2004.
7. Clinical practice guidelines for the use of tumor markers in breast and colorectal cancer: Adopted on May 17, 1996 by the American Society of Clinical Oncology. *J Clin Oncol*, v. 14, p. 2843-77, 1996.
8. Fitzgibbons, P. L. *et al*. Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med*, v. 124, p. 966-78, 2000.
9. McLaren, B. K. *et al*. Loss of expression of transforming growth factor beta type II receptor correlates with high tumor grade in human breast in situ and invasive carcinomas. *Am J Surg Pathol*, v. 29, n. 1, p. 105-8, 2005.
10. Gobbi, H. *et al*. Breast cancer risk associated with estrogen receptor expression in epithelial hyperplasia lacking atypia and adjacent lobular units. *Int J Cancer*, v. 113, p. 857-9, 2005.
11. Gouvêa, A. P. *et al*. Her-2/ neu immunoreactivity in invasive mammary carcinomas: a comparative study using monoclonal and polyclonal antibodies including the Herceptest. *J Bras Patol*, v. 40, n. 1, p. 47-52, 2004.
12. Hanna, W. *et al*. The predictive value of ERICA in breast cancer recurrence: a univariate and multivariate analysis. *Mod Pathol*, v. 6, p. 748-54, 1993.
13. Harvey, J. N. *et al*. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol*, v.17, p. 1474-81, 1999.
14. Huang, Z. et al . Novel rabbit monoclonal antibody to progesterone receptor (clone SP2): no heat pretreatment but effective for paraffin section immunohistochemistry. Appl Immunohistoch Mol Morphol, v. 14, p. 229-33, 2006.
15. Huang, Z. *et al*. Development of new rabbit monoclonal antibody to estrogen receptor: immunohistochemical assessment on formalin-fixed, paraffin-embedded tissue sections. *Appl Immunohistochem Mol Morphol*, v. 13, p. 91-5, 2005.
16. Jacobs, T. W. *et al*. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. *J Natl Cancer Inst*, v. 88, p. 1054-59, 1996.
17. McGuire, W. L. Breast cancer prognostic factors. Evaluation guidelines. *J Natl Cancer Inst*, v. 83, p. 154-1, 1991.
18. Pellicer, E. M.; Sundblad, A. Evaluation antibodies to estrogen receptors. *Appl Immunohistochem*, v. 2, p. 141, 1994.
19. Pertschuk, L. P. *et al*. Estrogen receptor immunocytochemistry in paraffin embedded tissues with ER1D5 predicts breast cancer endocrine response more accurately than H222Sp gamma in frozen sections or cytosol-based ligand-binding assays. *Cancer*, v. 77, p. 2514-19, 1996.
20. Rhodes, A. *et al*. Immunohistochemical demonstration of oestrogen and progesterone receptors: correlation of standards achieved on 'in house' tumours with that achieved on external quality assessment material in over 150 laboratories from 26 countries. *J Clin Pathol*, v. 53, p. 292, 2000.
21. Rhodes, A. *et al*. Study of interlaboratory reliability and reproducibility of estrogen and progesterone receptor assays in Europe. *Am J Clin Pathol*, v. 115, p. 44-58, 2001.
22. Rhodes, A. *et al*. Evaluation of HER-2/neu immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumours: a comparative study involving results from laboratories in 21 countries. *Am J Clin Pathol*, v. 118, p. 408-17, 2002.
23. Romain, S. *et al*. on behalf of the EORTC Receptor Study Group. EORTC Receptor Study Group Report: steroid receptor distribution in 47, 892 breast cancers. A collaborative study of 7 European laboratories. *Eur J Cancer*, v. 31A, p. 411-17, 1995.
24. Rossi, S. *et al*. A comparative study between a novel category of immunoreagents and the corresponding mouse monoclonal antibodies. *Am J Clin Pathol*, v. 124, n. 2, p. 295-302, 2005.
25. Sannino, P.; Shousha, S. Demonstration of estrogen receptors in paraffin wax sections of breast carcinoma using the monoclonal antibody 1D5 and microwave oven processing. *J Clin Pathol*, v. 47, p.90-2, 1994.
26. Stierer, M.; Rosen, H.; Weber, R. Immunohistochemical and biochemical measurement of estrogen and progesterone receptors in primary breast cancer: correlation of histopathology and prognostic factors. *Ann Surg*, v. 218, p. 13-21, 1993.
27. Treaba, D. O. *et al*. Significantly improved sensitivity for ER detection in breast cancer using a new rabbit monoclonal anti-ER antibody (SP1). *Mod Pathol*, v. 18, p. 53A, 2005.
28. Vassallo, J. *et al*. Comparison of immunoexpression of 2 antibodies for estrogen receptors (1D5 and 6F11) in breast carcinomas using different antigen retrieval and detection methods. *Appl Immunohistochem*, v. 12, p. 177-82, 2004.

Mailing address

Rafael Malagoli Rocha
Departamento de Anatomia Patológica, Faculdade de Medicina – UFMG
Av. Alfredo Balena, 190, sala 5.000
CEP: 30130-100 – Belo Horizonte-MG