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Tese

Anticorpos monoclonais contra receptor HER2: produção, caracterização e avaliação para uso em testes diagnósticos de tumores de mama humano e canino

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Anticorpos monoclonais contra o receptor HER2: produção, caracterização e avaliação para uso em teste diagnósticos de tumores de mama humano e canino

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RESUMO

VASCONCELLOS, Flávia Aleixo. **Anticorpos monoclonais contra receptor HER2: produção, caracterização e avaliação para uso em testes diagnósticos de tumores de mama humano e canino.** 2011. 67 f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

As neoplasias malignas, como o câncer de mama, atraem a atenção em todo o mundo por sua alta prevalência e grande demanda de recursos financeiros, e por representarem um grande ônus social. No Brasil, as taxas de mortalidade por câncer de mama continuam elevadas, muito provavelmente porque a doença ainda é diagnosticada em estágios avançados. Marcadores tumorais podem ser úteis no diagnóstico, no estadiamento e nas avaliações da resposta terapêutica e do prognóstico da doença. A superexpressão do oncogene *Human Epidermal growth factor Receptor 2* (HER2), um marcador de prognóstico em câncer de mama, é evidenciada em 20-30% dos tumores mamários em humanos e caninos. Nesse contexto, o presente trabalho relata o desenvolvimento de novos anticorpos monoclonais murinos apartir de uma proteína recombinante correspondente à porção extracelular do receptor HER2 para uso em técnica de imunohistoquímica (IHQ) e desenvolvimento de outros ensaios imunodiagnósticos. Estes anticorpos monoclonais foram produzidos, caracterizados e posteriormente testados em cortes histológicos de tumores mamários humano e canino. Entre cinco hibridomas produzidos, dois deles demonstraram potencial para uso na técnica de imunohistoquímica nestes tumores.

Palavras-chave: Anticorpos monoclonais. HER2. Imunohistoquímica. Câncer de mama.

ABSTRACT

VASCONCELLOS, Flávia Aleixo. **Anticorpos Monoclonais contra receptor HER2: produção, caracterização e avaliação para o uso em testes diagnósticos de tumores de mama humano e canino.** 2011. 67 f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia Universidade Federal de Pelotas, Pelotas.

Malignant neoplasms such as breast cancer deserve attention for its high prevalence and great demand of financial resources, representing a major problem of public health all over the world. In Brazil, mortality rates from breast cancer remain high, most likely because the disease is diagnosed in advanced stages. Tumor markers can be useful in diagnosis, staging and in the evaluations of therapeutic responsiveness and disease prognostic. The overexpression of the oncogene Human Epidermal growth factor receptor 2 (HER2), a prognostic tumor marker in breast cancer, is observed in 20-30% of breast cancers in humans and canines. In this context, this study reports the development of new murine monoclonal antibodies from a recombinant protein corresponding to the extracellular portion of the HER2 receptor for use in immunohistochemistry (IHC) and in the development of other immunoassays. These antibodies were produced, characterized and tested subsequently in histological sections of human and canine breast tumors. Among five hybridomas produced, two demonstrated potential for use in immunohistochemistry of these two tumors.

Keywords: Monoclonal antibody. HER2. Immunohistochemistry. Breast cancer.

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1 INTRODUÇÃO GERAL

Câncer é o nome dado a um conjunto de doenças que têm em comum o crescimento desordenado de células, que invadem tecidos e órgãos, estas células tendem a ser muito agressivas e incontroláveis, determinando a formação de tumores malignos (INCA, 2011).

O câncer é um importante problema de saúde pública em todos os países do mundo. Segundo o relatório da Agência Internacional para Pesquisa em Câncer (IARC)/OMS *(World Cancer Report 2008)*, o impacto global da doença mais que dobrou em 30 anos. O câncer é importante por sua alta prevalência, por consumir grande volume de recursos financeiros, por representar um grande ônus social, e por sua crescente relevância como causa de morte. Contudo, suas implicações podem ser reduzidas por meio de avanços que possibilitem a intervenção nos fatores de risco, no diagnóstico precoce e no tratamento (INCA, 2011; JEMAL et al., 2011).

No Brasil, as estimativas para o ano 2012, e que são válidas também para o ano 2013, apontam para a ocorrência de 518.510 casos novos de câncer. Os tipos mais incidentes, à exceção do câncer de pele do tipo não melanoma, são os cânceres de próstata e pulmão no sexo masculino, e de mama e colo do útero no sexo feminino (INCA, 2011).

Os dados do Instituto Nacional do Câncer (INCA) indicam que na população feminina brasileira o câncer de mama é o tipo mais incidente, correspondendo a 52.680 casos novos de câncer da mama, com um risco estimado de 52 casos a cada 100 mil mulheres. Já entre as mulheres norte-americanas, a probabilidade de desenvolver câncer de mama em algum momento da vida é de uma em cada oito mulheres (13%), e o risco de óbito decorrente deste tipo de tumor é de uma em cada três (33%) mulheres (JEMAL et al., 2008).

O câncer de mama é uma doença heterogênea, apresentando grande variabilidade clínica, patológica e molecular. Sua etiologia pode ser influenciada por fatores exógenos como dieta, consumo de álcool e uso de contraceptivos orais, entre outros, e por fatores endógenos como taxas hormonais e predisposição genética (RADICE e REDAELLI, 2003; INCA, 2011; JEMAL et al., 2011). Outros fatores de risco que influenciam no aparecimento desta doença incluem: a idade avançada, a menarca precoce (antes dos 12 anos), a menopausa tardia (após os 50 anos), a nuliparidade ou a primeira gravidez após os 30 anos, a localização geográfica (país de origem) e o *status* sócio-econômico (INCA, 2011; JEMAL et al., 2011).

Fatores prognósticos e preditivos são muito utilizados no manejo do câncer de mama. Fatores prognósticos são achados clínicos, patológicos e biológicos do paciente e do tumor que são úteis para predizer a evolução natural da doença. Por outro lado, os fatores preditivos são achados clínicos patológicos e biológicos usados para estimar a resposta, do paciente, a um tipo especifico de terapia (CIANFROCCA e GOLDSTEIN, 2004).

O prognóstico do câncer de mama é relativamente bom se diagnosticado no estágio inicial, evidenciando-se após cinco anos uma sobrevida média global em torno de 60%, na população mundial (INCA, 2011). Entre os fatores prognósticos tradicionais estão o acometimento de linfonodos axilares, o tamanho do tumor, o tipo e grau histológico, o grau nuclear, os níveis de progesterona e estrógeno, a velocidade de proliferação tumoral e a competência imunológica do portador da doença (BRASILEIRO FILHO, 2000; VIANA, MARTINS e GEBER, 2001). A identificação de marcadores moleculares por imunohistoquímica (IHQ) é utilizada no diagnóstico precoce do câncer de mama, sendo cada vez mais usuais na prática clínica (ESTEVA et al. 2002; ARCIERO et al. 2003; ESTEVA e HORTOBAGYI, 2004). Estes marcadores fornecem os valores preditivos e prognósticos, em associação com outros dados clínicos, para a tomada de decisão terapêutica (CIANFROCCA e GOLDSTEIN, 2004).

A maior parte dos casos de tumor de mama pertence à classe dos carcinomas, os quais se originam na região final dos ductos e associam-se às estruturas acinares, as quais são designadas de unidade terminal ducto-lobular,

consideradas unidades funcionais da mama (WÄRRI, SAARINEN e MÄKELÄ, 2007). Clinicamente, este tipo de câncer pode apresentar desde formas não invasivas, ou “in situ”, cuja proliferação celular está restrita à membrana basal, até formas em estágios mais avançados, ou invasivas, cuja proliferação invade a membrana basal e o tecido adjacente, e até formas altamente metastáticas, atingindo outros tecidos (NAKHLIS e MORROW, 2003; YUAN, SAHIN e DAI, 2005).

Os tumores mamários são neoplasias freqüentes também nas fêmeas caninas e representam um problema importante em medicina veterinária, visto que resultam em um alto índice de mortalidade (FIGHERA et al., 2008; CASSALI et al., 2009). Os tumores mamários que acometem os caninos compartilham diversas similaridades com os tumores da mama humana (GERALDES, GARTNER e SCHMITT, 2000; LEE, RUTTEMAN e KONG, 2004), sendo por isso considerados bons modelos para estudos comparativos (PAOLONI e KHANNA, 2008).

Entre os tratamentos para o câncer de mama, encontram-se as cirurgias (tumorectomia, quadrantectomia, mastectomia), a radioterapia e a quimioterapia. Recentemente, a imunoterapia e a modulação da resposta biológica têm sido usadas com resultados promissores (VIANA, MARTINS e GEBER, 2001; VALABREGA, MONTEMURRO e AGLIETTA, 2007). Entretanto, cerca de 50% dos casos de câncer de mama em humanos são diagnosticados tardiamente no Brasil, o que dificulta muito o tratamento e a cura e gera mais sofrimento ao paciente (INCA, 2011).

O diagnóstico do câncer de mama geralmente resulta da combinação de vários métodos como o auto-exame, o exame clínico palpatório realizado pelo médico, a mamografia, a termografia de placa e a ecografia. Uma biópsia deve então ser feita para confirmar o diagnóstico e identificar os marcadores moleculares que ajudarão a determinar o tipo específico de câncer e os tratamentos específicos (VIANA, MARTINS e GEBER, 2001; CHODOSH, 2011)

Os receptores de estrógeno e de progesterona, e o oncogene HER2 (de *Human Epidermal growth factor Receptor 2*), são marcadores moleculares que possuem um reconhecido valor preditivo no câncer de mama, sendo ultilizados para avaliar, respectivamente, a resposta à terapia hormonal e ao tratamento com o anticorpo monoclonal (DUFFY, 2005; CASSALI et al., 2011). Além disso, a utilização

na prática clínica de outros marcadores moleculares de prognóstico, por exemplo, o gene supressor de tumor p53, o receptor beta de ácido retinóico, o fator de crescimento derivado de plaquetas (PDGF) e o fator de crescimento vascular endotelial (VEGF), entre outros, ainda está sob investigação (ESTEVA et al., 2002; ARCIERO et al., 2003; KEEN e DAVIDSON, 2003; ESTEVA e HORTOBAGYI, 2004; ZHANG e YU, 2011).

A proteína HER2 está superexpressada em aproximadamente 20% a 25% dos carcinomas ductais de mama, mas estes índices podem aumentar para 40% em casos de pacientes com invasão de linfonodos. Essa proteína apresenta um alto grau de similaridade com outros membros da família de receptores tirosina-quinase (ISHII et al., 1987; XIE et al., 2000; RABINDRAN, 2005). Em geral, mulheres com carcinoma invasivo HER2 positivo possuem uma doença mais agressiva, com uma probabilidade maior de reincidência, um pior prognóstico e uma sobrevida menor, quando comparado com as mulheres HER2 negativo (YAMAUCHI, STEARNS E HAYES, 2001; TIMMS et al., 2002; HENRY e HAYES, 2006).

Ahern et al. (1996), avaliaram a expressão do gene *HER2* no nível do RNA mensageiro em tecidos tumorais de mama canino e verificaram que 75% dos 23 carcinomas estudados apresentaram superexpressão. Posteriormente, outros estudos utilizando a IHQ com critérios similares para avaliação da coloração verificaram que 17% a 35,4% dos carcinomas na espécie canina apresentavam aumento da expressão da proteína (RUNGSIPIPAT et al., 1999; MARTÍN DE LAS MULAS et al., 2003; DUTRA et al., 2004), sugerindo que a HER2 pode ser um importante fator para carcinogênese mamária canina como ocorre na espécie humana. Contraditoriamente, a superexpressão de HER2 foi relacionada com maior sobrevida dos animais (HSU et al., 2009).

O oncogene HER2 em humanos localiza-se no braço longo do cromossomo 17q12-21 e codifica uma glicoproteína transmembrânica (receptor) de 185-KDa, a qual possui atividade tirosina-quinase em sua porção carboxi-terminal (AKIYAMA et al., 1986). Na espécie canina o gene HER2 é localizado no cromossomo 1q13.1 (MURUA ESCOBAR, BECKER e BIELLERDICK, 2001).

Os receptores com atividade de tirosina quinase que atuam como mediadores primários no processo de sinalização celular, regulando a progressão do ciclo

celular, rearranjo do citoesqueleto, diferenciação celular e apoptose (CASALINI et al., 2004; ROSKOSKI, 2004). A subclasse I da família de recelptores tirosina quinase é constituída por receptores de fatores de crescimento epidérmico e compreende quatro membros: EGFR/HER1; HER2; HER3 e HER4. Nos tumores de mama, as alterações moleculares nestes receptores ocorrem frequentemente no HER2 e geralmente associam-se à superexpressão do mesmo (KRAUS et al., 1987; STERN, 2000; HYNES e LANE, 2005).

Os heterodímeros formados pelo HER2 possuem um potencial de sinalização extremamente elevado (CASALINI et al., 2004). No entanto, quando o receptor HER2 encontra-se superexpresso, a presença de um ligante para que a sua heterodimerização com os outros receptores da sua família, ou que sua homodimerização ocorra, não se faz necessária (OLAYIOYE, 2001). Isto explica a capacidade desse receptor em desencadear a ativação constante de uma via de transdução de sinal, mesmo quando nenhum ligante específico está presente (ROSKOSKI, 2004).

A amplificação do gene *HER2* e/ou a superexpressão da proteína HER2 caracteriza uma fração bastante significativa dos carcinomas de mama e, por essa razão, esse oncogene tem sido alvo para o diagnóstico através de várias técnicas como: Imunohistoquímica (SLAMON et al., 1989; SINGLETON et al, 1992; ONITILO et al, 2009), *Southern* (SLAMON et al, 1987, 1989) e *Slot blotting* (KALLIONIEMI et al., 1992), ELISA (DITTADI et al., 1997, 2001) e Hibridização *in situ* (PRESS et al., 1997; ROSS e FLETCHER, 1999; SCHALLER et al, 2001). Assim sendo, novas abordagens terapêuticas que visam combater as células que superexpressam HER2 estão sendo desenvolvidas (RÉVILLION, BONNETERRE e PEYRAT, 1998; ZHOU e HUNG, 2003; RABINDRAN, 2005; ZHANG e YU, 2011).

Testes errôneos ou inconclusivos para HER2 podem resultar em inapropriada conduta clínica e irão dificultar a tomada de decisão sobre a utilização de novas drogas para o tratamento do câncer de mama (DENT e CLEMONS, 2005). Vem daí a busca por anticorpos específicos, como no caso dos anticorpos monoclonais e ainda a diminuição do valor de mercado para uso em pesquisa ou até mesmo laboratório clínico, já que em sua maioria, os anticorpos utilizados no Brasil, atualmente, para fins diagnósticos, são importados, agravados ainda pela sua

produção em animais de experimentação, quando diz respeito a anticorpos policlonais (NUNES et al., 2007), diferente dos anticorpos monoclonais, que uma vez produzidos, o hibridoma estável produz por tempo indeterminado o anticorpo monoclonal desejado (KOHLER e MILSTEIN, 1975).

Nesse contexto, o presente trabalho, descreve o desenvolvimento de novos anticorpos monoclonais em murinos contra uma proteína recombinante proveniente de um fragmento do cDNA, correspondendo a porção extracelular do receptor HER2 (residuos 76 ao 309), que codifica a proteína HER2, para uso em técnica de imunohistoquímica e desenvolvimento de outros ensaios imunodiagnósticos. Estes anticorpos foram caracterizados e testados em cortes histológicos de tumor mamário humano e animal, através da técnica de IHQ. As lâminas coradas por cada anticorpo foram submetidas à avaliação individual, seguindo-se o sistema de *score* recomendado pela *American Society of Clinical Oncology/College of American Pathologists Guideline* (WOLFF et al., 2007).

No primeiro artigo intitulado “Monoclonal antibodies 410G and 33F against human and canine HER2 protein”, relatamos a produção e caracterização, através da metodologia de imunização, fusão, seleção clonal, isotipagem, especificidade e aplicabilidade de dois hibridomas anti-HER2. O artigo foi publicado na revista *Hybridoma* volume 30, Number 5, 2011. DOI: 10.1089/hyb.2011.0066.MAB

No segundo artigo intitulado “Generation and characterization of new HER2 monoclonal antibodies”, relatamos a produção de cinco anticorpos monoclonais produzidos contra a proteína recombinante obtida apartir de um fragmento do gene HER2, a caracterização deles através de ELISA, *Western Blot* com a proteína recombinante e com a proteína na sua forma nativa usando as células MCF7, uma linhagem de células de câncer de mama, pela técnica de imunofluorescência e com cortes de tecido tumoral de mama humano pela técnica de imunohistoquímica. Além disso, caracterizamos os anticorpos monoclonais produzidos quanto ao seu potencial de uso, pelas técnicas de constante de afinidade e isotipagem. O segundo artigo foi submetido ao periódico *Scandinavian Journal Immunology.*

No terceiro artigo intitulado “Detection of HER2 overexpression in clinical or canine mammary tumor: A preliminary study with a novel monoclonal antibody”, relatamos a aplicabilidade dos anticorpos monoclonais anti-HER2 produzidos, no

diagnóstico imunopatológico em carcinomas mámarios de caninos, demonstrando através da técnica de imunohistoquímica o potencial de uso dos MAbs em medicina veterinária. O terceiro artigo foi submetido ao periódico *Veterinary Immunology and Immunopathology.*

2 ARTIGO 1

Artigo publicado no Periódico *Hybridoma*
(ANEXO1)

Monoclonal antibodies 410G and 33F against human and canine HER2 protein

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3 ARTIGO 2

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Generation and Characterization of New HER2 Monoclonal Antibodies

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Generation and Characterization of New HER2 Monoclonal Antibodies

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Running Title: Monoclonal antibodies against HER2

Abstract

HER2 oncoprotein is overexpressed or undergoes gene amplification in approximately 20 to 30% of invasive breast cancers. HER2 positive breast cancer is associated with a poor prognosis, higher rate of disease recurrence and increased resistance to chemotherapy and hormonal therapy. However, patients with HER2 positive breast cancer can benefit from adjuvant therapies that target this receptor. Overexpression of HER2 is routinely detected by immunohistochemistry (IHC) on tissue sections from fixed tumor blocks. We describe the production of five novel stable murine hybridoma clones secreting monoclonal antibodies (MAbs) that react with the HER2 protein. For hybridoma production, spleen cells from BALB/c mice immunized with a recombinant fragment of the extracellular portion of HER2 (rHER2) were fused to SP2/O-Ag14 cells, selected in HAT medium and screened in an indirect ELISA using rHER2. MAbs secreted were characterized according to isotypes, functional affinity constants, and ability to react with the native protein in MCF-7 cells by immunofluorescence (IF) and in tissue sections from a HER2 positive breast cancer specimens by immunohistochemistry (IHC). Two MAbs were of the IgG2b and three were of the IgG1 isotypes, and their affinity constants ranged from $6x10^{7}$ to $1x10^{9}$ M^{-1}. All MAbs reacted with the native protein in MCF-7 cells and two (clones 410G and 33F) stained strongly the membrane of tumor cells overexpressing HER2. These MAbs could be useful in assaying HER2 overexpression in breast cancers by IHC and in the study of the role of this protein in other types of cancers.

Keywords: HER2; Monoclonal antibodies; Breast cancer; MCF7; Immunohistochemistry; Immunofluorescence**;**

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2, ERBB2) is a 185 kDa transmembrane protein encoded by a gene located on chromosome 17q21. It exhibits tyrosine kinase activity and is involved in the development of several types of cancers including breast, esophagus, stomach, pancreas and bladder [1,2]. HER2 gene amplification or protein overexpression is detected in approximately 20–30 percent of patients with invasive breast cancer [3]. HER2 positive breast cancer patients have a poor prognosis (higher rate of recurrence and mortality) if not treated with adjuvant systemic therapy, which demonstrates the prognostic clinical significance of the gene status. Another even more important aspect of HER2 determination is its role as a treatment predictive factor. HER2 positivity predicts response to anthracyclin and taxane based therapies and shows relative resistance to hormone therapies such as Tamoxifen®. Moreover, HER2 overexpression enables the use of selective HER2-targeted therapy with the humanized monoclonal antibody Trastuzumab® as a neo-adjuvant or adjuvant chemotherapy agent in these patients [4-6].

HER2 status strongly affects therapeutic decisions and is now routinely evaluated in all newly diagnosed cases of invasive breast carcinoma. HER2 testing methods of choice are immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) techniques on formalin fixed and paraffin-embedded neoplastic tissue [7]. IHC semiquantitatively detects HER2 protein overexpression, is easy to perform, and is relatively inexpensive. However, this technique can be influenced by a variety of factors related to assay protocols. Pre-analytical (e.g. tissue fixation) and

analytical (e.g. testing protocol, and type of antibody) can modify HER2 immunoreactivity and affect the results interpretation. *In situ* hybridization techniques, specifically FISH, quantify the amount of HER2 gene amplification and are considered to be more accurate and reliable than IHC. They are not widely available and are technically more difficult to perform in the laboratory [8,9].

Several studies have shown high concordance rates between IHC and FISH assay results in the negative (0/1+) and positive (3+) IHC expression categories, with no need for dual IHC/FISH testing in the majority of cases [10-13]. In this context, the current strategy to identify breast cancer patients expected to benefit from those who would not from HER2 targeted therapy is to use IHC as a primary screening tool and to select equivocal cases (2+) for testing for gene amplification by FISH [7]. Currently there are two mouse (CB11 and 4D5) and one rabbit (4B5) antibody clones being widely used in HER2 evaluation by IHC. Because equivocal IHC results have been attributed to differences in sensitivities and specificities of these antibodies [14-17] , it is important to search for novel antibodies which would more accurately predict gene amplification by *in situ* hybridization. To this end, we have generated a panel of five novel monoclonal antibodies (MAbs) to HER2 and characterized them with regard to isotype, affinity to a HER2 extracellular domain recombinant fragment, immunofluorescence analysis of a breast cancer cell line (MCF-7), and IHC staining of tissue sections from known positive and negative invasive breast carcinomas previously tested with other anti-HER2 antibodies.

MATERIALS AND METHODS

Recombinant Protein Preparation

A segment of the HER2 gene was amplified by PCR using a cDNA fragment (IMAGE human, Invitrogen, Calsbed, California, USA) corresponding to the extracellular portion of the HER2 receptor (residues 76 to 309). The amplicon was cloned into the pQE30 expression vector, which allows fusion of the protein with a 6×His tag, resulting in the plasmid named pQE/HER2. This plasmid was used to transform *E. coli* BL21(DE3)Star and cultivated in Luria-Bertani liquid medium containing 100 µg/mL of ampicillin. When the absorbance at 600 nm reached 0.8, 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the recombinant protein expression. After 3 hours of induction, the culture was subjected to centrifugation at 10 000 × *g* for 5 min at 4 °C, the supernatant discarded, and the pellet subjected to two successive washes in 60 mL of PBS under the same centrifuging conditions. The washed pellet was then suspended in 60 mL of lysis buffer (8 M urea, 20 mM Na2HPO4, 0.5 M NaCl, 5 mM Imidazole). The cells were lysed by six cycles of sonication (15 s, 20 kHz) and submitted to orbital shaking of 60 rpm for 16 hours at 4 °C. After this, the cells were centrifuged again at 10 000 × *g* for 1 hour at 4 °C, the supernatant containing the protein was collected, filtered through a membrane filter of 0.8 µm (Millipore) and used for purification. The purification of recombinant protein was done by affinity chromatography using HisTrap™ HP 1 mLl columns prepacked with precharged Ni Sepharose™ using the ÄKTAprime™ automated liquid chromatography system (GE Healthcare, Piscataway, NJ, USA). Fractions containing the recombinant protein (rHER2) were diluted in AKTA wash buffer without urea containing 0.2% *N*-lauroyl sarcosine (1:20), dialyzed against Tris-

HCl 100 mM, pH 8.0, with 150 mM NaCl for approximately 16 hours at 4 °C and concentrated with PEG. rHER2 protein in the final preparation was analyzed by 15% SDS-PAGE, Western blot and quantified by the Bradford method [18].

Cell Line

The human breast cancer cell line MCF-7 (a gift from Dra Fátima Tereza Alves Beira, Departamento de Fisiologia, Universidade Federal de Pelotas, Brazil) was cultured in modified Eagle's medium (MEM; Gibco, Invitrogen, Calsbed, California, USA) supplemented with 10% heat-inactivated fetal calf serum (CULTILAB, Campinas, SP, Brazil), 2 g/L sodium bicarbonate and 1% antibiotic-antimycotic solution (Gibco, Invitrogen, Calsbed, California,USA).

Immunization of Mice and Generation of MAbs

Six week-old female BALB/c mice were injected intraperitoneally (IP) on Day 1 with 250 µL of a solution containing 70 µg of rHER2 protein mixed in an equal volume of Freund's complete adjuvant. Injections were repeated on Days 14, 21 and 28 using Freund´s incomplete adjuvant. Indirect ELISA was used to verify immunization efficiency, and mice showing the highest anti-rHER2 titer received a final IP booster injection. Three days after the last booster, splenocytes were removed and fused to Sp2/OAg14 myeloma cells in the presence of PEG 1450. Hybridomas were selected

under HAT medium and positive wells were identified using indirect ELISA with rHER2 as the capture antigen. The cells from wells presenting antibody activity were cloned twice by the limiting dilution technique, retested, and cultivated for freezing in liquid nitrogen or injecting in mice previously primed with pristane for ascites production. MAbs were purified from ascitic fluid using a protein A-Sepharose 4B column according to the manufacturer instructions (GE Healthcare, Piscataway, NJ, USA), dialyzed against PBS and concentrated with PEG 20000. The concentration of purified MAbs was determined by the Bradford method and the preparations were stored at -20°C until use.

Isotyping

ELISA was used to determine the MAbs isotypes with specific commercially available anti-isotype sera (Sigma-Aldrich, St Louis, MO, USA). Briefly, 3 µg/mL of rHER2 were added to microtitre plate wells coated with carbonate-bicarbonate buffer, pH 9.6, and incubated for 1 hour at 37°C. The wells were washed three times with PBST (PBS plus 0.05% Tween 20) and hybridoma culture supernatants were added and left to react for 1 hour at 37 °C. The washing was repeated and the isotype specific sera diluted 1:2000 were added and incubated for 30 minutes at room temperature (RT). The washing was repeated and a goat anti-mouse peroxidase conjugate diluted 1:5000 was added and incubated for 1 hour at 37 °C. Wells were washed five times with PBST and the presence of the antigen-antibody complex was revealed with a substrate/chromogen solution containing OPD (0.4 mg/mL in 0.1 M citrate buffer, pH 5.0) and 0.03% hydrogen peroxide. Color development was

measured at 450nm in an ELISA reader (Multiskan MCC/340, Titertek Instruments, Huntsville, AL, USA).

Affinity Constants

MAbs functional affinity constants (Ka) for rHER2 were determined by the ELISA method.[19] Briefly, concentrations of the antigen varying from 2.0 x 10^{-7} M to 1.8 x 10^{-9} M were incubated in solution with known amounts of each MAb until equilibrium was reached (16 hours at 30 ^{o}C). After incubation, unbound antibodies in the liquid phase were determined by indirect ELISA using microtiter plate wells coated with rHER2 (3 μg/mL) and goat anti-mouse Ig-peroxidase conjugate. The optical densities (OD) were read at 450 nm and graphs were built according to Bobrovnik [20]. The linear relationships between values $(A_0 - A_i)/A_i$ and I_i (where A_0 is the OD of MAb without reacting with antigen, A_i is the OD of MAb after reacting with each antigen concentration, and I_i is the antigen concentration) were determined and the values of functional Ka were defined by the slope of these linear relationships.

Indirect Immunofluorescence

Slide chambers (ICN Biomedicals Inc., Costa Mesa, CA, USA) were coated with a 0.01% poly-L-lysine solution (Sigma-Aldrich, St Louis, MO, USA) overnight at RT. A culture of MCF-7 was washed once in PBS, resuspended to a density of 6×10^{7} cells per milliliter in PBS, and incubated in the slide chamber for 1 hour at 30 °C. The slides were washed twice with PBS plus 2% BSA and fixed with methanol -20 °C for

10 min and coated with MAb preparations diluted 1:100 in PBS plus 2% BSA. After incubating for 1 hour at RT in a dark humid chamber, the slides were washed again twice with PBS plus 2% BSA and a 1:200 dilution of rabbit anti-mouse FITC conjugate (Sigma-Aldrich, St Louis, MO, USA) was added and incubated for 1 hour in a dark humid chamber at 30 °C. After washing with PBS plus 2% BSA, a 1:2000 dilution of Hoechst 33258 (Sigma-Aldrich, St Louis, MO, USA) was added and incubated for 1 hour in a dark humid chamber at 30 °C. After washing with PBS plus 2% BSA, a drop of mounting medium and a coverslip were added. The following controls were used in this experiment: (1) positive control: mouse polyclonal antibody against HER2 was used as the primary antibody; (2) negative control: normal mouse serum was used as the primary antibody. Labeling was visualized by fluorescence microscopy using an Olympus BX 51 microscope.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue from one HER2 positive (3+) and one HER2 negative (0/1+) breast cancer cases were tested with the MAbs. Four-micron thick sections were cut from a representative paraffin block and mounted on previously silanized glass slides. The slides were dried for 60 min at 60 ^{o}C and were left resting overnight at RT. The following day sections were deparaffinized with xylene and rehydrated with ethanol. Then, they were treated with citrate buffer (0.01 M citric acid, pH 6.0) and heat-based antigen retrieval was carried out in a hot bath at 92 ^{o}C for 30 minutes. Endogenous peroxidase was blocked using 95 mL of methanol plus 5 mL of 3% hydrogen peroxide solution. After that, preparations were washed in

distilled water and PBS solution. Unspecific protein binding was blocked with 5% non-fat dry milk diluted in PBS for 30 minutes. The primary antibodies used were: monoclonal antibodies anti-rHER2 produced in this study diluted 1:50, 1:100, and 1:200; mouse polyclonal antibody anti-rHER2 produced in this study diluted 1:50, 1:100, and 1:200; and polyclonal rabbit anti-human HER2 (clone A0485, DAKO, Denmark) diluted 1:200 and 1:400. The antibodies were incubated overnight at 4 ^{o}C on a level surface and in a dark humid chamber. The following day, sections were processed by the streptavidin-biotin-peroxidase technique using the LSAB kit/HRP (DAKO, Denmark) according to manufacturer directions. To reveal the reaction, a diaminobenzidine (DAB) solution (60 mg DAB diluted in 100 mL PBST, plus 1 mL H_2O_2 30V) was employed. The slides were then counterstained with hematoxylin, dehydrated and mounted with Entellan (Merck) for optical microscopy analysis (Olympus, model IX 71). Positive and negative controls were used throughout the assay.

RESULTS

Recombinant Protein

The rHER2 protein fragment was cloned and expressed in *E. coli*. It was expressed as inclusion bodies and required denaturing conditions for purification, followed by dialysis to obtain soluble protein preparations. After the purification process, preparations contained a protein with more than 95% purity that showed in

immunoblot analysis a band of 27.3 kDa corresponding to the gene fragment expressed (not shown).

MAbs Production and Isotyping

As result of a single fusion experiment, five stable hybridoma clones secreting MAbs against rHER2 were obtained. The clones named 18F, 13A, 410G, 16C, and 33F were of the IgG1 and IgG2b isotypes and yielded from 0.397 mg/mL to 2.73 mg/mL after purification from ascitic fluids (Table 1).

Affinity constants

The MAbs functional affinity constants determined by ELISA, average of three independent experiments, ranged from 6×10^{7} to 1×10^{9} M^{-1} (Table 1).

Immunofluorescence

The five MAbs stained MCF-7 cells by IFA. Specific fluorescence signals dispersed on the cell outer membrane were observed. No fluorescence staining was observed in the negative control (Figure 1).

Immunohistochemistry

The five MAbs were tested by IHC to demonstrate their ability to bind HER2 protein in neoplastic tissue from human breast cancer. Two MAbs (clones 410G and 33F) demonstrated strong complete membrane staining in more than 30% of the tumor cells (3+) (Figure 2). The other three MAbs did not demonstrate membranous staining or faint/ barely perceptible staining in other parts of the tumor cells (0/1+).

DISCUSSION

The detection of HER2 protein overexpression is a prerequisite for selection of patients with invasive breast carcinoma for treatment with the humanized monoclonal antibody Trastuzumab. Most pathology laboratories perform a preliminary selection of cases using IHC, due mainly to its rapidity and lower costs when compared with the *in situ* hybridization techniques. Although it is easier to perform, IHC has disadvantages that include subjectivity in evaluating the staining score, possible loss of HER2 protein as a result of tissue storage and fixation, and variable results depending on both the antibody and staining procedure used [21].

Several commercially available polyclonal and MAbs are routinely used for HER2 detection [17,22,23]. However, many of these antibodies cross-react with other tumor antigens or present a high background staining due to nonspecific reactions [24].

In this study, we report on the production of MAbs for a recombinant protein fragment (27.3 kDa) from the HER2 receptor. Five stable hybridoma clones secreting MAbs of the IgG isotype that reacted with epitopes from the native HER2 protein were obtained, as seen by IFA with MCF-7 cells and IHC with breast cancer specimens. The fact that they are of the IgG isotype and react with the native protein, suggest that the MAbs are well suited for use in diagnostic techniques such as IFA and IHC [24].

MAbs were further characterized regarding their affinity constants, which ranged from 6×10^{7} to 1×10^{9} M^{-1}. As expected, the two MAbs with the highest affinities (410G and 33F) were those with the best IHC results. A high affinity antibody is likely to react faster with the tissue antigen and give a more intense IHC staining within the same incubation period than a low affinity antibody [25].

The IFA carried out with the breast cancer cell line MCF-7 yielded only a weak staining of cell outer surface due to the fact that these cells express HER2 protein at basal levels on their plasma membrane. Despite this, the results were sufficient to confirm that the MAbs have potential for use in IFA, since they all reacted with the MCF-7 cells.

IHC technique using the MAbs and citrate-steamed tissue sections of a specimen of paraffin-embedded breast cancer tumor overexpressing HER2 revealed that MAbs 410G and 33F strongly labeled the plasma membrane with low background reaction, while the other three did not. These results, together with IFA results, suggest that the antigen retrieval treatment used may not be adequate to reveal epitopes for the other three MAbs. IHC is frequently influenced by technical considerations, such as

fixation and antigen retrieval procedures and differences in methodological protocols [24,26]. Besides, the selection of specific antibodies and scoring methods are very important for the accurate evaluation of protein expression [17].

In conclusion, we have generated five murine MAbs that react with epitopes in native HER2 protein. Two of these five MAbs could be useful reagents for use in diagnostic methods such as IFA and IHC as well as for other research purposes.

DISCLOSURE/CONFLICT OF INTEREST

None declared.

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Figure Legends

Figure 1. Immunofluorescence of MCF-7 cells with antibodies against HER2. A- Hoescht + mouse polyclonal anti-HER2; B- FITC + mouse polyclonal anti-HER2; C- Hoechst + normal mouse serum; D- FITC + normal mouse serum; E- Hoechst + MAb 410G; F- FITC + MAb 410G.

Figure 2. Immunohistochemistry in neoplastic tissue from human breast cancer (x100). A- Negative control (normal mouse serum 1:50); B- Positive control (1:200 rabbit polyclonal anti-human HER2, clone A0485, DAKO®); C- MAb 410G anti-HER2 1:200; D- MAb 33F anti-HER2 1:50.

Figure 1

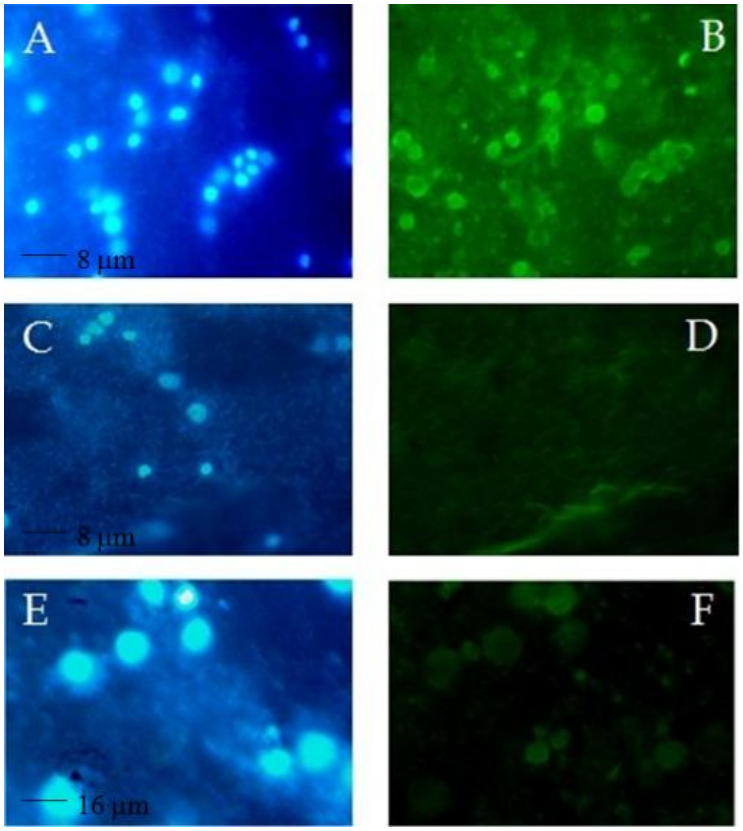

Figure 2

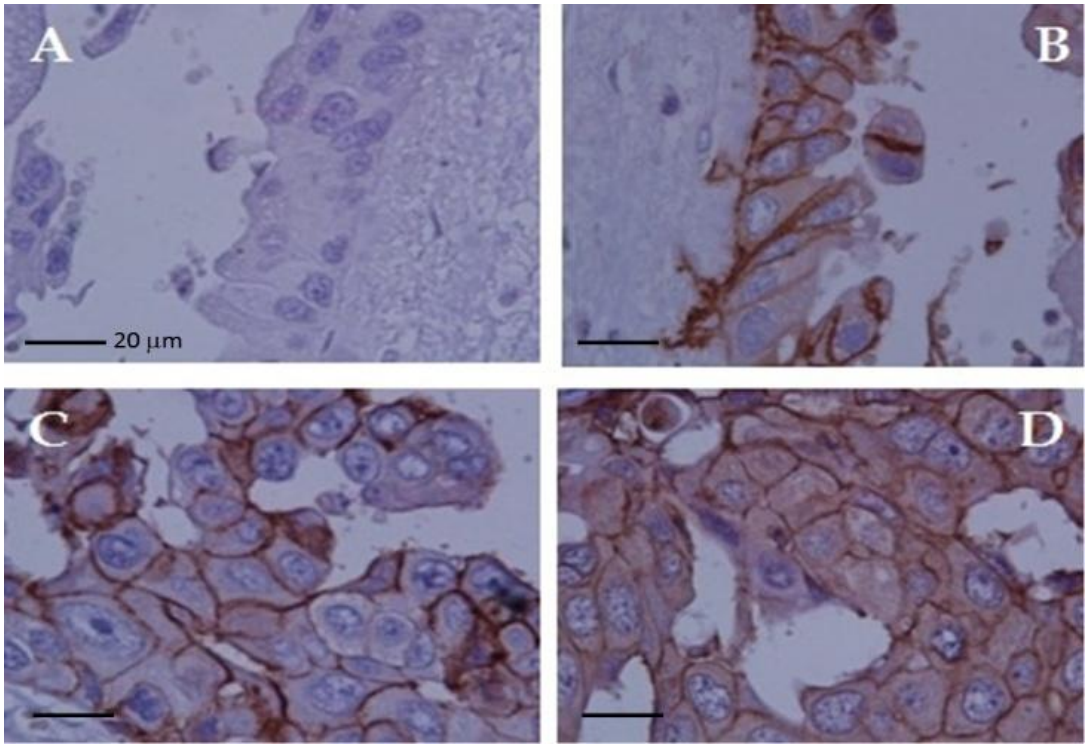

Table 1

Isotypes, affinity constants and yield of MAbs against HER2.

Monoclonal Antibody	Isotype	Affinity Constant ($L.mol^{-1}$)	Yield[a] ($mg.mL^{-1}$)
33F	IgG_1	$6x10^8$	0.39
16C	IgG_{2b}	$8x10^8$	2.73
18F	IgG_{2b}	$1x10^8$	1.39
410G	IgG_1	$1x10^9$	1.12
13A	IgG_1	$6x10^7$	1.52

[a]From ascitic fluid

4 ARTIGO 3

Artigo formatado segundo as normas do Periódico
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(Short Communication)

Detection of HER2 overexpression in clinical cases of canine mammary tumor: A preliminary study with a novel monoclonal antibody

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Short Title: HER2 overexpression in canine mammary tumor

Abstract

Canine mammary tumors have similarities with human disease, becoming an applicable model for comparative purposes. The HER2 (Human Epidermal Growth Factor Receptor 2) has an important role in mammary gland and its overexpression is correlated to a poor prognostic in mammary carcinoma. In this study, we investigated the applicability of a monoclonal antibody against HER2 protein, which was generated and characterized by our group, in tissue sections using a semi-quantitative immunohistochemical (IHC) assay to determine the overexpression of HER2 protein in eight canine mammary carcinoma samples which were previously confirmed by clinical diagnostic routine. As major result, mAb 33F stained strongly more than 30% of the tumor cells overexpressing HER2, presenting a similar performance in the IHC test when compared with the commercial antibody. Our findings confirm that mAb 33F could be useful in assaying HER2 overexpression in breast cancers by IHC and in the study of the role of this protein in other types of cancers.

Keywords: Monoclonal antibody / HER2 / immunohistochemistry / canine breast cancer

1. Introduction

Human breast cancer is the most frequently diagnosed cancer and cause of death in women worldwide (Jemal et al., 2011). In pet animals cancer is a major cause of death (Withrow, 2007), and mammary tumors in female dogs represent the most prevalent type of neoplasms (Kumaraguruparan et al., 2006). The incidence of mammary carcinomas in female dogs is three times that documented in humans (Sorenmo, 2003), and according to the

previous studies between 41 and 53% of the canine mammary tumors are considered malignant (Misdorp et al, 1999; Rutteman et al., 2001; Moe, 2001).

Canine mammary tumors have similarities with human disease, becoming an applicable model for comparative purposes (Molina et al., 2005). Immunohistochemical markers for early detection of mammary tumors have been studied and ER, HER2, CK5, p63, and P-cadherin have already been identified in canine carcinomas and demonstrated similarity between both species (Gama et al., 2008).

HER2 overexpression was observed in 20–30% of human breast cancers (Almasri and Al Hamad, 2005), and in 17.6-29.7% of malignant canine mammary tumors (Rungsipipat et al., 1999; Hsu et al., 2009). Previous studies showed that either HER2 amplification (Ahern et al., 2006) or HER2 protein overexpression (Rungsipipat et al., 1999; Martin de las Mulas et al., 2003) were present in canine mammary tumors. Moreover, studies have demonstrated that HER-2 overexpression is associated with an adverse prognosis (Ross et al., 2003). Thus immunohistochemistry can still be useful to adapt the information gained from high throughput technologies for clinical diagnosis.

The objective of the present investigation was to evaluate a novel murine monoclonal antibody against HER2 by immunohistochemistry assay, using tissue of canine mammary tumours.

2. Material and Methods

2.1 *Tumors tissue samples*

Tumors tissue samples were obtained by excisional biopsy from eight female dogs admitted to Hospital das Clínicas Veterinárias (HCV/UFPel) for clinical evaluation and treatment of mammary tumors (Table 1). Tumor size was determined by the pathologist on the gross specimen. Tissues were fixed in 10% formalin in phosphate-buffered saline pH 7.6, until use.

2.2 Monoclonal Antibody

The clone named 33F belonging to IgG1 isotype against rHER2 was obtained from the Centro de Desenvolvimento Tecnológico / Núcleo de Biotecnologia- Laboratório de Imunologia Aplicada, Universidade Federal de Pelotas, Brazil (Vasconcellos, 2011). For all experiments, the concentration was adjusted to approximately 500 µg/mL.

2.3 *Immunohistochemistry Assay*

Immunohistochemistry assay for HER2 was performed using the LSAB kit/HRP (DAKO, Denmark). Serial 3µm sections were cut from normal canine mammary tissue and from tumor samples. Paraffin was removed by treatments in xylene and sections were hydrated in absolute ethanol. Block endogenous peroxidase with methanol/hydrogen peroxide 3% rehydrated ethanol. Briefly, after epitope retrieval in sodium citrate buffer (pH 6.0, 10mM) at 95-99°C in microwave. The HER-2 immunostaining protocol includes overnight incubation in primary monoclonal antibody 33F at a dilution of 1 in 100 and rabbit polyclonal antibody A0485 (Dako) diluted 1 in 200. Rinse sections and incubate with secondary reagent biotinylated. Rinse slides and incubate with estreptavidin-HRP2. Detection of immune

reaction with developing reagents (3,3'- diaminobenzidine tetrahydrochloride chromogen solution), hematoxylin counterstain and mounted. As a positive control we used human breast cancer tissue known to express HER2; negative controls were prepared by using normal serum as the primary antibody. The criteria for staining intensity were based on scoring system: negative (score 0): no staining or membrane staining in <10% of tumour cells; weak (score 1+): faint/largely incomplete membrane staining in >10% tumour cells; moderately positive (score 2+): weak to moderately complete membrane staining in >10% tumour cells; strongly positive (score 3+): Strong complete membrane staining in >10% tumour cells. In this study, scores 0 and 1+ were assigned as normal expression of HER-2 protein and scores 2+ and 3+ were grouped as overexpression of HER-2 protein. Sections were observed and photographed using an Eclipse E400 microscope (Nikon, Tokyo, Japan).

3. Results and discussion

Table 1 shows the information about clinical and epidemiologic data of the female dogs. Canine breeds included four Poodle, one Fila, one Yorkshire, and two Cross breed, and the mean age was 7.75 years (range 5-11 years), and just one (12.5%) of them had received hormonal contraception treatment. During examination in HCV/UFPel, macroscopically carcinomas were well delineated and solid masses measuring range to 0.5–11.0 cm of diameter. HER2 test analyses performed using rabbit polyclonal antibody A0485 (Dako) revealed that five (62.5%) of animals reacted positively (2+ or 3+), and three animals were negative in the test. Predominant growth pattern in histological analyses showed lesions in varying as micropapillary, tubulopapillary, mixed cell malignant and connective tissue components, among others (Table 1).

In our study, the novel mAb 33F (Vasconcellos, 2011) derived from a single B-cell clone and produced by hybridoma technique provide excellent specificity when tested by immunohistochemistry (IHC) to demonstrate their ability to bind HER2 protein in neoplastic tissue from canine breast cancer. The clone 33F demonstrated strong complete membrane staining in more than 30% of the tumor cells (3+) (Fig.1). Our results were similar to previous results of HER2 overexpression detection in immunopathological diagnostic assay performed in mammary tissue samples of HCV/UFPel (Table 1).

Assessment of HER2 status has gained increased importance in the clinical management of patients with breast cancer (Wang, 2000). Recent studies have demonstrated that 50% of canine mammary tumours are malignant and most of them metastasize (Millanta et al., 2005). HER-2 overexpression has been identified in 20–30% of human breast cancers and of malignant canine mammary tumors (Almasri and Al Hamad, 2005; Hsu et al., 2009).

Morphological diagnosis in veterinary medicine has classically relied mostly on routine stains such as hematoxylin and eosin, and less commonly on other histochemical stains (Ramos-Vara et al., 2008). However, with the increasing prevalence of canine cancer, the level of specialization in veterinary practice demands more accurate diagnosis, the search for biomarkers such as overexpression of HER2 oncogene correlate with disease prognosis particularly for tumors before treatment starts (Dagli, 2008).

Immunohistochemistry (IHC) has been proven to be one of the most important ancillary techniques in the characterization of neoplastic diseases in humans and has become equally important in veterinary medicine, as oncologists demand more specific diagnoses. The number of immunohistochemical tests offered by veterinary diagnostic laboratories for the diagnosis of infectious and neoplastic diseases in frozen or formalin-fixed, paraffin-embedded (FFPE) tissues has increased exponentially in the last decade (Ramos-Vara et al., 2008). On

the other hand, inconsistencies in the sensitivity of IHC HER2 testing have been attributed to the use of different antibodies, differences in tissue fixation and IHC reagents, antigen retrieval methods and interobserver variability (Thomson et al., 2001; Press et al., 2002; Wolff et al., 2006).

In summary, we demonstrated the excellent performance of a new murine monoclonal antibody directed against HER2 protein in the determination of the HER2 status in breast cancer canine by immunohistochemistry.

Conflict of interest statement

The authors declare that they have no conflict interests.

Author's Contributions

All authors designed the experimental protocols and participated in sampling. FAV wrote most of the manuscript and did the experimental execution and analysis together with JBR and SCS. EFS and JAGA contributed to writing, data presentation and supervision. All authors read and approved the final manuscript.

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Figure 1

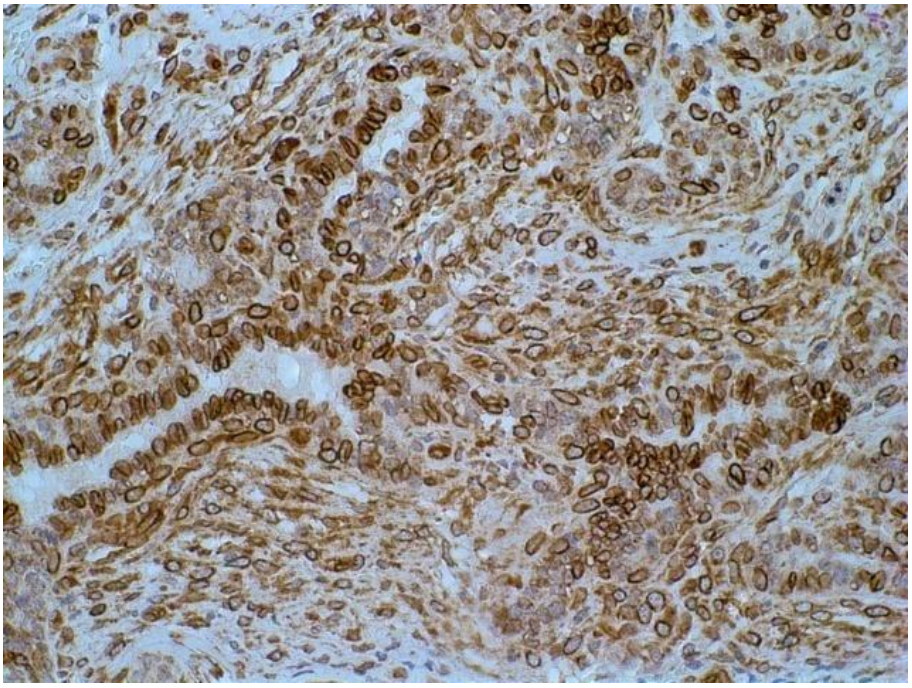

Figure 1 Immunohistochemistry in neoplastic tissue from canine breast cancer with MAb 33F anti-HER2, 1:100 (x40).

Table 1 Clinical and pathological data

Case	Breed	Age (years)	Contraception	Nodule size (cm)	Predominant growth pattern	HER2 Test A0485	HER2 Test 33F	Follow up
1	Cross breed	11	NA	6.0	Micropapillary	P (3+)	P (3+)	Dead after 1 month
2	Fila	9	NA	11.0	Papillary-cystic	P (2+)	P (2+)	Did not recover from surgery
3	Yorkshire	7	YES	3.0	Mixed cell malignant epithelial and connective tissue components	P (3+)	P (3+)	Euthanased after 5 months
4	Poodle	8	NA	4.0	Tubulopapillary	N (0)	N (0)	Lost to follow-up
5	Poodle	6	NA	1.5	Epithelial component of malignant and benign myoepithelial	N (0)	N (0)	AW after 10 months
6	Poodle	5	NA	0.5	Tubulopapillary	P (+2)	P (+2)	Dead after 28 month
7	Poodle	8	NA	4.0	Solid with formations micropapillary	P (+3)	P (+3)	Lost to follow-up
8	Cross breed	8	NA	5.0	Epithelial component of malignant and benign myoepithelial	N (0)	N (0)	AW after 18 months

(NA, Data not available; AW, Alive and Well; P, Positivo; N, Negativo)

5 CONCLUSÕES

- Os cinco anticorpos monoclonais produzidos (33F, 410G, 13A, 18F, 16C) reconheceram o receptor HER2, os quais foram purificados e caracterizados; os hibridomas foram armazenados e serão usados para dar continuidade aos trabalhos de validação dos anticorpos em ensaios imunoquímicos de detecção da superexpressão de HER2 em tumores mamários e outras neoplasias malignas.

- Os anticorpos monoclonais 33F e o 410 G demonstraram potencial para detecção da superexpressão da proteína do receptor HER2 em cortes histológicos de tumor de mama humano através da técnica de imunohistoquímica.

- O anticorpo monoclonal 33F possui capacidade de detecção da superexpressão da proteína do receptor HER2 em tecido de carcinoma mamário canino através da técnica de imunohistoquímica.

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7 ANEXO

7.1 ANEXO 1

HYBRIDOMA
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Monoclonal Antibodies 410G and 33F Against Human and Canine HER2 Protein

Antigen Used for Immunization

A segment of the *HER2* gene was amplified by PCR using a cDNA fragment (IMAGE human, Invitrogen, Carlsbad, CA) corresponding to the extracellular portion of the HER2 receptor (residues 76-309). The amplicon was cloned into the pQE30 expression vector. This plasmid was used to transform *E. coli* BL21(DE3) Star. The purification of recombinant protein was done using the ÄKTAprime™ automated liquid chromatography system (GE Healthcare, Piscataway, NJ). The rHER2 protein in the final preparation was analyzed by 15% SDS-PAGE and quantified by the Bradford method.

Method of Immunization

Six-week-old female BALB/c mice were injected intraperitoneally with 70 µg of rHER2 protein in Freund's complete adjuvant. Injections were repeated on days 14, 21, and 28 using Freund's incomplete adjuvant. Mice were boosted intravenously with approximately 10 µg of antigen in saline 3 days before fusion.

Parental Cell Line Used for Fusion

Sp2/0-Ag14 myeloma cells (ATCC# CRL-1581).

Selection and Cloning Procedure

Hybridomas were selected under HAT medium and positive wells were identified using indirect ELISA with rHER2 as the capture antigen. The cells from two wells presenting high antibody activity were cloned twice by the limiting dilution technique, retested, and cultivated for freezing in liquid nitrogen.

Heavy and Light Chains of Immunoglobulin

Both antibodies are IgG1. Light chains were not determined.

Specificity

The MAbs stained MCF-7 cells by immunofluorescence. Specific fluorescence signals dispersed on the cell outer membrane were observed. No fluorescence staining was observed in the negative control cells.
The MAbs were tested by immunohistochemistry and were found to be effective on binding HER2 protein in formalin-fixed, paraffin-embedded tissue sections from human[1] and canine breast cancer.[2]

Specific Antigen Identified

Human and canine HER2 protein.

Application

Immunoassays for detection of HER2 protein.

Availability

Tissue culture supernatant	Yes ✓	No
Ascitic fluid	Yes	No ✓
Hybridoma cells	Yes	No ✓

References

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2. Vasconcellos FA, Stone SC, Raposo JB, Conceição FR, Silve ÉF, and Aleixo JAG: A monoclonal antibody against recombinant human HER2 protein detects HER2 overexpression in canine breast cancer. In: XXV Congress of the Brazilian Society for Immunology. Porto Alegre, Brazil, 2010.

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