The Value of a New Cancer Biomarker fHER-2 Proto-oncogene in the Diagnosis of Feline Mammary Carcinoma

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Abstract. The overexpression of the Human Epidermal growth factor Receptor-2 (HER-2) oncogene in human breast cancer is associated with a poor prognosis and a specific treatment. Because of its importance and as a first line option for diagnosis, well established guidelines for its detection are based in immunohistochemical techniques, Still, in Veterinary Medicine there is little and inconsistent information about this subject. The aim of our study was to achieve an optimal immunohistochemical protocol for detection of *f*HER-2 in Feline Mammary Carcinoma (FMC). Five commercial anti-HER-2 antibodies were tested using three different protocols. The *f*HER-2 protein overexpression was detected in 10 of the 30 FMC cases (33.3%), when the optimized protocol was performed (associating the A0485 antibody with a longer antigen retrieval method). These results suggest that *f*HER-2 may play an important role in Feline Oncology and that the Cat can be a suitable animal model for human breast cancer research.

1 Introduction

The HER-2/neu proto-oncogene encodes a 185kD transmembrane glycosylated protein that belongs to the human epidermal growth factor receptor's family [10]. In humans, this gene is located on chromosome 17 and its amplification, identified in 20 to 30% of breast cancers, is an important diagnostic and prognostic marker [9]. In most of the cases, HER-2 gene amplification leads to an increase in protein expression levels which results in an increase number of HER-2 receptors in the cell membrane. Because it is clinical relevance, evaluations of HER-2 status by immunohistochemical (IHC) and by in situ hybridization assays were recently validated by the American Society of Clinical Oncology (ASCO). Also in last years, Gentech/Roche companies engineered a humanized monoclonal antibody that inhibits the receptor's dimerization providing a longer survival period in breast cancer patients [12].

In Feline Oncology, the mammary tumors are very common. Indeed, they are the third most common tumor in clinical practice and represent 17% of the tumors in female cats. Feline Mammary Carcinomas (FMC) have display some particularities that distinguish them from the dog mammary tumors. They are very aggressive (85%

Soares M., Correia J., Cabeçadas J., Peleteiro C. and Ferreira F..
The Value of a New Cancer Biomarker fHER-2 Proto-oncogene in the Diagnosis of Feline Mammary Carcinoma.
DOI: 10.5220/0003879300090017
In *Proceedings of the International Workshop on Veterinary Biosignals and Biodevices* (VBB-2012), pages 9-17
ISBN: 978-989-8425-94-2

are malignant), showing a poor prognosis and a short survival period [15]; [3]; [4]. Recently, some studies had revealed a wide *frequency* range of mammary tumors *f*HER-2+ (5%-90%) using one or three commercial antibodies [1]; [14]; [10]. Also, the role of *f*HER-2 in oncogenic mechanisms remains unknown.

In this study we aim to improve the immunodetection of *f*HER-2 in order to obtain a better evaluation of HER-2 status on formalin-fixed, paraffin wax-embedded tissue sections of FMC. For this we used 5 different commercial antibodies (two of them never used in feline samples) and three different antigen retrieval (AR) methods. In the end, we intend to contribute for the characterization of the frequency of FMC-*f*HER-2 and compare our results with others authors, for a better understanding
Beyond the potential applications in Veterinary Medicine, the study of the status of this oncogene could have clinical relevance, while cats can be a suitable natural model for studying human HER-2 positive breast cancer.

Finally, we also point out the future contributions that Engineering Sciences can bring to improve the *f*HER-2 immunodetection and the immunotherapy of FMC's.

2 Material and Methods

2.1 Sample Collection and Histology

The 30 mammary gland samples used in this study were obtained from the Anatomical Pathology Diagnostic Service archives, Faculty of Veterinary Medicine, Lisbon, Portugal and complemented with clinical information provided for each case. These mammary specimens were fixed in formaldehyde and embedded in paraffin blocks. Only samples of carcinomas fixed for less than 72 hours were considered for the study (Table 1). For histologic examination, sections of 4μm thickness were stained with haematoxylin-eosin (HE) and tumors were classified according to the World Health Organization (WHO) criteria [7].

Table 1. Histologic classification according to the WHO and grading of the samples submitted to immunohistochemical evaluation.

Histologic classification	Malignant Grade	Samples (%)	Total
Cribiform carcinoma	II	1/30 (3.3%)	11/30 (36.7%)
	III	10/30 (33.4%)	
Tubulopapillary carcinoma	II	4/30 (13.3%)	9/30 (30%)
	III	5/30 (16.6%)	
Tubular adenocarcinoma	II	2/30 (6.6%)	6/30 (20%)
	III	4/30 (13.3%)	
Mucinous carcinoma	III	1/30 (3.33%)	4/30 (13.3%)
Simple Carcinoma	I	1/30 (3.33%)	
Solid carcinoma	III	1/30 (3.33%)	
Squamous cell carcinoma	III	1/30 (3.33%)	

2.2 Immunohistochemical Study

HER-2/neu Antibodies. Five commercial antibodies were tested for *f*HER-2/neu immunostaining on paraffin tissue sections: a rabbit polyclonal anti-human HER-2 (A0485 from DAKO, Glostrup, Denmark), two rabbit monoclonal anti-human HER-2 antibodies (4B5 from Ventana, Tucson, Arizona and SP3 from Zytomed, Berlin, Germany) and two mouse monoclonal anti-human HER-2 antibodies (CB11 from Zytomed and TAB250 from Invitrogen, Carlsbad, California). Each one of these antibodies has literature showing that they recognize the HER-2 protein in human tissues by binding to extracellular domain of the HER-2 receptor (TAB250 and SP3) or to recognize the intracellular domain (CB11, 4B5 and A0485). We also note that SP3 and TAB250 antibodies were used for the first time in order to detect *f*HER-2 in this work.

Immunohistochemical Technique. Sections were mounted on Starfrost® microscope slides and dried at 60ºC for one hour. Each slide were deparaffinized and rehydrated in distilled water through a series of graded alcohols and then submitted to antigen retrieval with buffer citrate solution ($NaCH_3COO$, pH=6) in a water bath at 95ºC for 30 minutes or for 60 minutes as resumed in Table 1I. In parallel, to improve antigen recognition of TAB250 antibody, we performed an enzymatic digestion of tissue samples with Protease K (Zymed) for 10 minutes following manufacturer's recommendations. To exhaust endogenous peroxidase activity, a Peroxide-Block solution (Zytomed) was applied for 10 minutes and each primary antibody was incubated during 1h at room temperature. After several PBS washes, primary antibodies were detected with a secondary antibody for 30 minutes (HER2*easy* kit IHC from Zytomed) and 3,3'-diaminobenzidin-tetrahydrochlorid (DAB) was used as the chromogen prior to counterstain with Mayer's haematoxylin.

Positive and negative controls were obtained from human breast carcinomas known to overexpress HER-2 receptor and previously classified as 3+ or, classified as 0 without HER-2 expression (see Table 3).

Table 2. Resume of the immunohistochemical protocols used for *f*HER-2 detection.

Primary antibody			Antigen retrieval
Clone	Dilution	Incubation time	
CB11	RTU	60'	Buffer citrate solution 95º C for 30' and 60'
4B5	RTU	60'	
A0485	1:250	60'	
SP3	1:100	60'	
TAB250	1:50	60'	Buffer citrate solution 95º C for 30' and 60' Proteinase K for 10'

RTU = Ready to use

Interpretation Criteria. Overexpression of *f*HER-2 was defined as a membranous staining in more than 10% of neoplastic cells and staining was examined over the maximum area of staining intensity according to the DAKO guidelines (Table 3).

Samples classified as 0 or 1+ were considered negative, whereas scores of 2+ or 3+ were considered positive. Cytoplasmic staining was considered nonspecific staining. All slides were submitted to blind scoring by two independent DVM pathologists and one DVM clinician. Any discordant interpretation was debated and settled using a multiviewer microscope.

Table 3. Interpretation Criteria (HercepTest Interpretation Manual from DAKO).

Grade	Interpretation
0	No staining.
1+	Weak, incomplete membranous staining in any proportion of tumor cells.
2+	Complete membrane staining that is either no uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells.
3+	Uniform intense membrane staining of at least 10% of invasive tumor cells.

2.3 Statistical Study

The association between *f*HER-2 overexpression and grade of malignancy or histological classification were assessed by Fisher's Exact Test. Values of $p < 0.05$ were considered to reflect statistical significance.

3 Results

The mean age of the 30 queens at the time of mastectomy was 10.4 years. Cribiform carcinoma (36.7%) was the most common type of malignant mammary tumor, followed by the tubulopapillary (30%) and tubular carcinomas (20%). The histologic grading reveals that almost all of these tumors (73.3%) were poorly differentiated carcinomas, showing a grade III.

The immunodetection of *f*HER-2 by some commercial antibodies (CB11, 4B5 and A0485) was revealed by a cellular membrane labeling in several FMC showing a species cross reactivity. Positive (3+) and negative (0) controls show the label intensity expected in all protocols (Figure 1).

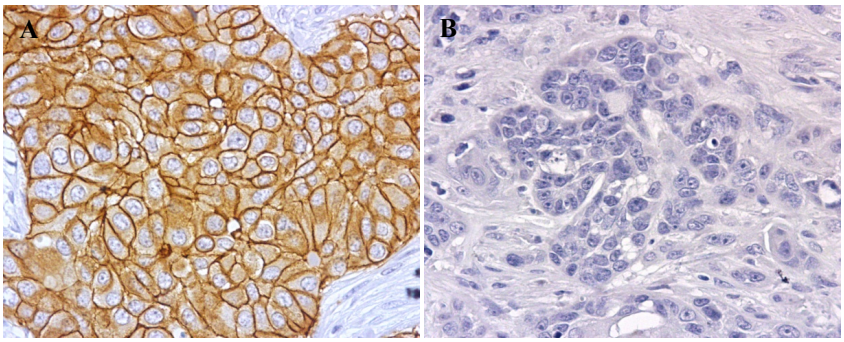

Fig. 1. Images of positive and negative controls using a CB11 antibody after 60' of antigen retrieval. **(A)** Human positive control scored 3+ showing an intensive and continuous label of cellular membrane (x400); **(B)** Human negative control scored 0, with no staining (x400).

Results obtained from the employment of TAB250 and SP3 antibodies showed no staining in all samples even after use a longer antigen retrieval protocol and in regards of the others, the best results were achieve with A0485, as its shown in Figure 2.

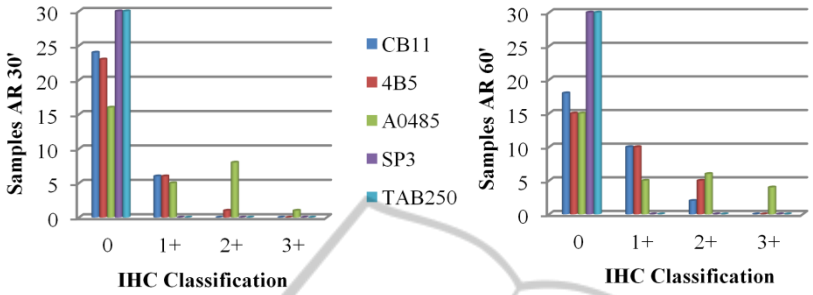

Fig. 2. Results of IHC protocols using two antigen retrieval durations (30' or 60'). Note that regardless the protocol used, there are no staining for SP3 and TAB250. It's also clear the improvement in the results with 60' of antigen retrieval in all the remaining three antibodies. **(A)** Scores of IHC with the lower antigen retrieval (30 minutes); **(B)** Scores of IHC with the longer antigen retrieval (60 minutes).

Table 4 summarizes our results, in which we observe a *f*HER-2 overexpression in 6.7% of the samples using CB11 antibody, 16.7% with 4B5 antibody and 33.3% with polyclonal antibody A0485 from DAKO, when we use a longer antigen retrieval method (Figure 3). When compared, all samples that demonstrated overexpression with CB11 or 4B5 had the same or a better score with A0485.

Table 4. IHC results using the CB11, 4B5 and A0485 as primary antibodies.

IHC Classification		**0**	**1+**	**2+**	**3+**	**TOTAL**
Antibody / AR method						
CB11	AR 30'	24 (80%)	6 (20%)	0 (0%)	0 (0%)	30 (100%)
	AR 60'	18 (60%)	10 (33.3%)	2 (6.7%)	0 (0%)	30 (100%)
4B5	AR 30'	23 (76.7%)	6 (20%)	1 (3.3%)	0 (0%)	30 (100%)
	AR 60'	15 (50%)	10 (33.3%)	5 (16.7%)	0 (0%)	30 (100%)
A0485	AR 30'	16 (53.3%)	5 (16.7%)	8 (26.7%)	1 (3.3%)	30 (100%)
	AR 60'	15 (50%)	5 (16.7%)	6 (20%)	4 (13.3%)	30 (100%)

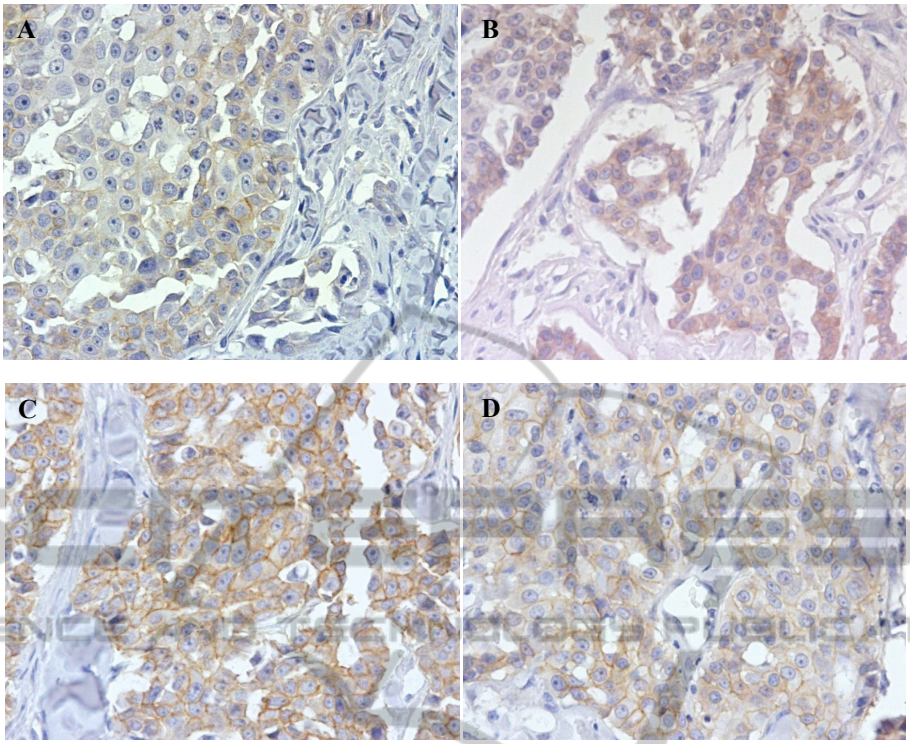

Fig. 3. Expression of *f*HER-2 in the same sample, classified as Cribiform Carcinoma **(A)** Score classification 1+ using CB11 and 60'AR (x400). **(B)** Score classification 1+ using 4B5 and 60'AR (x400). **(C)** Score classification 3+ using A0485 and 60'AR (x400). **(D)** Score classification 2+ using A0485 and 30'AR (x400).

The 4B5 antibody was the one that showed more cytoplasmic staining, with samples with diffuse homogeneous staining in the cytoplasm and sometimes with dot artifacts. Also a weak to moderate non-specific cytoplasmic labeling was seen in the dermal adnexal structures (Figure 4).

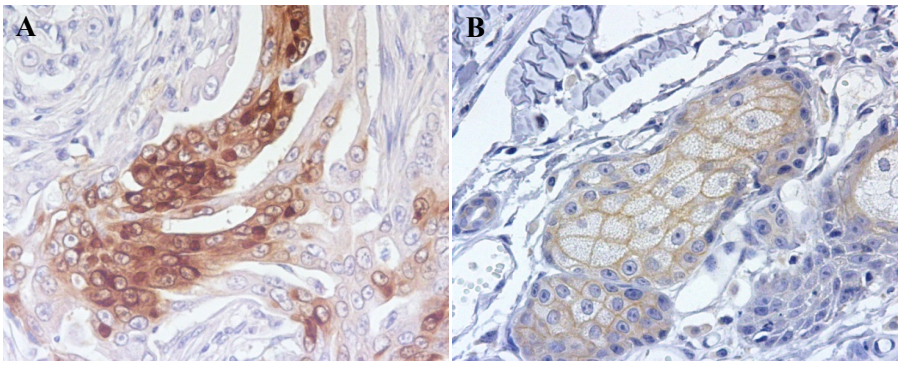

Fig. 4. (A) Cribiform carcinoma scored 1+ with 4B5 and 30' AR showing homogeneous no specific cytoplasmic staining (400x); **(B)** Strong staining of a dermal adnexal structure, a sebaceous gland (x400).

When analyzed, *f*HER-2 overexpression in FMC did not evidenced significant correlation with histological classification (p-value = 0.28) neither with malignance grade (p-value = 0.47).

4 Discussion

In the past decades we have seen an increased attention and development of companion animals health services, which create new needs that led to obvious improvements and findings in Veterinary Medicine. If for one hand this made our pets more resistant to diseases and with larger/bigger life-expectancy, on the other hand it urged the rise of pathologies usually confined to the geriatric population, where the neoplasms fit. Our animals share the same lifestyle as humans and start to be seen as potential models to research, especially for Cancer Research.

In Cancer Research, the Molecular Biology has a fundamental role, and research in this area continues to rise, with new information and discoveries published every day. Unfortunately, in Veterinary Medicine there is little information available on molecular alterations and the biological behavior of tumors in our companion animals. In FMC, it is usual to request the histological and grading classification, but the proteomic status of some receptors isn't routinely performed.

In the present study we compared five different antibodies anti-HER-2, and we have found different results, with the most promising one being the A0485, mostly when combined with a longer antigen retrieval method than the time suggested by the manufacturer (preferably 60 minutes AR duration), where we obtained an overexpression of 33.3%, a result similar to those published for Human, with ranges between 10 to 40% [5], [16].

For human patients, ASCO guidelines are very specific and with well defined exclusion criteria. Among these recommendations the fixation is imperative and all the samples fixed in fixatives other than neutral buffered formalin should be excluded. Beyond this, the samples must be fixated for longer than 6h and less than 48hours [16]. As our samples did not fulfilled all these requirements we can not exclude that this may have influenced the results, especially for the SP3 and TAB250 antibodies, that recognize the extracellular domain, which could be more affected by the inadequate fixation and as consequence did not show any staining; nor can we disregard the influence of fixation for the discrepant results obtained among the three antibodies recognizing the intracellular domain.

The CB11 and the 4B5 antibodies are monoclonal while the A0485, with best results, is a polyclonal antibody. If we associate the fact that the first two antibodies that recognize a human epitope of the HER-2 protein, with a aminoacid sequence homology of 93% (when compared to cat) and, the evidence that the latest antibody recognizes several human epitopes of the same protein we can suspect that the wide range of results (6.6% to 33.3%) that were obtained can be due, besides the fixation problems, to the not total homology between the HER-2 and *f*HER-2, which makes A0485 more suitable to recognize the protein.

We can also easily conclude that the antigen retrieval method is critical in the immunohistochemical assessment of HER-2 in feline tissues and that when this step is shorter (30' instead of the 60'), it may significantly lower the threshold of positivity.

These findings are contrary to those of a recent study, where they found a decreased positivity with most prolonged antigen retrieval [10].

If we compare our findings with the results present by other authors, the incidence of *f*HER-2 overexpression is similar to some of the studies [1]; [7]; [10] but markedly lower than in other reports [6]; [14]. Besides the interpretation criteria in one of these studies being different to the one we have used [14] the other have respected the tissue proceeding guidelines [6], which reinforces the importance of this step, so often neglected. Indeed, in the majority of Diagnostic Services of Anatomical Pathology, it is usual to receive samples that do not fulfill the requisites for a correct immunohistochemical analysis. So, it is important to sensitize the clinicians and the surgeons for this problem moreover since the histological classification and malignant grade show to be insufficient to classify the tumors and, because none of them demonstrated predictive value for determination of *f*HER-2 status in our studies, which is concordant with others publications [6], [10].

The possibilities to use and introduce engineering sciences to improve the fHER-2 evaluation and anti-fHER-2 clinical treatments were studied. However, two extra obstacles would have to be passed to achieve the total optimization of *f*HER-2 immunodetection in feline mammary tumors, whereas engineering can give an important contribution. One of them is the automatization absence of the technique which leads to different results between different laboratories and the other is the interobserver subjectivity in scoring the HER-2 expression in formalin-fixed, paraffin-embedded breast cancer tissues, due to a very high cellular heterogeneity and to an extensive calcification/necrotic tumor areas [13], [2].

In human oncology, the American Society of Clinical Oncology (ASCO) recommends the use of standardized operating procedures, the validation of laboratories methodologies and also suggests that two or more expert pathologists should score independently the same patient's tissue sample to avoid wrong classifications. To minimize the variability of the results and enhance the reproducibility, automated systems were developed recently for human samples, where all the steps of the technique can be regulated (from the deparaffinization of the tissues till the mounting of slides). Also very recently, a new Automated Cellular Imaging System (ACIS®, Clarient ChromaVision Medical Systems) was announced to standardize the detection, the counting and the classification of tumor cells based on recognition of cellular bodies with a specified shape, size and color.

In the near future, we think that Engineering Sciences can bring a substantial contribution by developing/adapting the automatized devices similar to the ones used in the human tissues processing. Additionally, we see as a very promising tool the improvement of the adjunctive computer-assisted methodology to feline mammary carcinomas samples, providing reproducibility in the acquisition and scoring of immunohistochemical images evaluated by a qualified pathologist, after the development of new image processing algorithms.

Acknowledgements

The authors thank Dr. Rita Ribeiro for the statistical analysis, Dr. João Matos and Dr.

Sandra Carvalho from the Anatomical Pathology Service. This work was supported by CIISA and FCT (SFRH/BD/70720/2010).

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