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Changes in c-erbB-2 Immunoexpression in Feline Endometrial Adenocarcinomas

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Contents

Human epidermal growth factor receptor type 2 (c-erbB-2), an oncoprotein with potential prognostic marker and therapeutic use, is overexpressed in several human and animal tumours. But information regarding this molecule in feline tumours is scarce. This study aimed to assess the changes in the immunohistochemical expression of c-erbB-2 in feline endometrial adenocarcinomas (FEA) compared to normal endometrium. An immunohistochemistry assay using a specific antibody against c-erbB-2 was performed in FEA samples (n = 34) and in normal endometrium in the follicular (FS; n = 12) and luteal (LS; n = 11) stages. In FEA, the c-erbB-2 immunoexpression was assessed in neoplastic epithelial cells whilst in normal endometria it was individually evaluated in the surface and the superficial and deep glandular epithelia (SE, SGE and DGE, respectively). In FS and in LS, all the epithelia were positive for c-erbB-2; positivity was higher in the SE and the SGE than in DGE. Twenty of the 34 FEA samples (58.8%) were positive for c-erbB-2 immunolabelling. Nevertheless, its expression was higher in all the epithelia in the FS compared to FEA (p ≤ 0.0001) or the LS (p = 0.016). The results presented herein suggest that c-erbB-2 molecule is differently expressed in the feline endometrium through the oestrous cycle and though it may also be involved in feline endometrial carcinogenesis, a question remains unanswered on the importance of additional pathways of epithelial proliferation in the neoplastic changes in feline endometrium.

Introduction

Human epidermal growth factor receptor type 2 (c-erbB-2) is overexpressed in several human cancers, including endometrial adenocarcinomas (Yarden and Sliwkowski 2001), and its amplification is often associated with tumour invasiveness, particularly in breast cancer (Brix et al. 2014). In women, the reported rates of c-erbB-2 overexpression in endometrial carcinomas vary from 1% to 80% depending on its histological type (reviewed by Buza et al. 2014). This very wide range of results is pointed as related to small sample sizes, different clinicopathological features considered and/or differences in the staining and the scoring systems used in immunohistochemistry evaluation of the tumours. For these reasons, it is possible that in human endometrial carcinomas c-erbB-2 overexpression and tumour prognosis and outcome may not always be correlated (Buza et al. 2014). On the other hand, an increased expression of c-erbB-2 is particularly well-studied in women mammary carcinomas (Yarden and Sliwkowski 2001). In c-erbB-2-overexpressing human breast carcinomas, targeted therapy against c-erbB-2 is proven to be effective. Therefore, standardization of c-erbB-2 assessment is well-established in these tumours (Wolff et al. 2013), unlike in endometrial cancer (Buza et al. 2014).

Scoring guidelines for c-erbB-2 evaluation by immunohistochemistry have been recently established for the canine mammary tumours (Peña et al. 2014), which are similar to those existing in humans. The evaluation of c-erbB-2 in feline mammary tumours has also been studied, but no similar guidelines exist (Millanta et al. 2005; Winston et al. 2005; Ordás et al. 2007; Rasotto et al. 2011). Several studies suggest c-erbB-2 overexpression in feline mammary carcinomas as a marker of malignancy and a prognostic indicator (Millanta et al. 2005; Winston et al. 2005; Ordás et al. 2007), but this is controversial, Rasotto et al. (2011) defending that c-erbB-2 may not play a major role in mammary carcinogenesis and prognosis. Technical factors and subjective interpretation of c-erbB-2 immunolabelling may explain the disagreements in the incidence of c-erbB-2 expression between studies (Rasotto et al. 2011).

Increasing reports in feline endometrial adenocarcinomas (FEA) (Gil da Costa et al. 2009; Anderson and Pratschke 2011; Cho et al. 2011; Payan-Carreira et al. 2013; Sontas et al. 2013; Saraiva et al. 2015a) suggest that the disease may be more common than previously described. The definitive diagnosis is usually accomplished by histopathological exam. Histopathological features of FEA, although not constant, include recognized criteria of malignancy such as nuclear atypia, bizarre mitoses, necrosis, and vascular, myometrium and/or serosa invasion (Papparella and Roperto 1984; Miller et al. 2003; Anderson and Pratschke 2011; Saraiva et al. 2012). Despite the growing number of studies in FEA, little is known about the molecular events related to the carcinogenesis of the endometrium in queens. Moreover, immunohistochemical characterization of FEA is limited and performed in small case series (Espinosa de los Monteros et al. 1999; Miller et al. 2003; Gil da Costa et al. 2009). Nevertheless, the pathogenesis of FEA remains elusive.

Considering that c-erbB-2 could play a role in feline endometrial carcinogenesis, the present study aimed to characterize the c-erbB-2 immunohistochemical pattern of expression in FEA compared to normal endometrium, and to explore putative roles of c-erbB-2 in feline cyclic and neoplastic endometrium.

Materials and Methods

Source of samples

The samples of FEA (n = 34) and of normal post-pubertal feline uterus (n = 23, 12 in the follicular stage – FS – and 11 in the luteal stage – LS) used in the present work were collected from the archives of four different laboratories in Portugal, during a period of 8 years (from 2003 to 2011). The age, breed, and past or present progestin-based contraception of animals enrolled in the study were assessed by the request forms accompanying the surgical specimens for histopathological evaluation. For FEA, the clinical history leading to the submission of specimens to the laboratories was also recorded.

Histopathological examination

Tissues were fixed in 4% buffered formalin and paraffin embedded. Sections with a thickness of 3 μm were cut, one slide routinely stained with haematoxylin and eosin for histopathological diagnosis. The tumours were evaluated according to several criteria of malignancy as described in Saraiva et al. (2015a). Briefly, the histopathological criteria used included potential indicators of tumours dedifferentiation, namely: nuclear atypia, considered as low to moderate or high; mean number of mitoses per high power field (HPF; 400x), valued as lower than 1 (<1), 1–5 and more than 5 (>5); and the myometrium, serosa and/or vascular invasion, evaluated as present or absent. Twenty-three normal uterine samples were staged as FS (n = 12) or LS (n = 11) based on the presence of follicles or corpora lutea in the ovaries and the epithelial cell height and the degree of development and coiling of endometrial glands (Saraiva et al. 2015a).

Immunohistochemistry

Immunohistochemistry was performed using a polymer-based system (Novolink® Polymer Detection System, Product No: RE7280-K Leica Biosystems, Newcastle, UK), according to the manufacturer's instructions. The sections were deparaffinized with xylene and rehydrated through graded alcohols; antigen retrieval was performed in a pressure cooker (~96°C) in 10 mmol/l sodium citrate buffer (pH 6.0) for 3 min. The slides were cooled for 10 min at room temperature and rinsed twice in triphosphate buffered saline (TBS) for 5 min. Endogenous peroxidase activity was blocked by treating the sections with the Peroxidase Block® provided in the kit. Sections were incubated overnight at 4°C in a humid chamber with 1 : 40 Polyclonal Rabbit Anti-Human [5] c-erbB-2 Oncoprotein® (Code A0485 Dako, Denmark) that labels an intracellular domain of c-erbB2 oncoprotein. Colour was developed with 3,3′- diaminobenzidine (DAB), incubated at room temperature and sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted. Sections of human colonic adenocarcinoma were used as positive control. For negative controls, the primary antibody was omitted.

Quantification of immunolabelling

The immunoreactivity of c-erbB-2 was assessed semi-quantitatively, in morphologically representative areas of the tumour at low power fields, based in the Hercep-Test scoring criteria, described in the American Society of Clinical Oncology (ASCO) guidelines (Wolff et al. 2013). Briefly, samples were classified according to the score: IHC3+, when the circumferential membrane staining is complete, intense and within >10% of tumour cells ('chicken-wire pattern'); IHC2+, if the circumferential membrane staining is incomplete and or weak/moderate but within >10% of tumour cells, or complete and circumferential membrane staining that is intense in only ≤10% of tumour cells; IHC1+, if incomplete membrane staining is faint/barely perceptible and within >10% of tumour cells; IHC0, no staining is observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤10% of tumour cells (Wolff et al. 2013). Scores of IHC0 and 1+ were considered negative, whereas scores of IHC2+ and 3+ were considered positive (Grushko et al. 2008; Rasotto et al. 2011; Togami et al. 2012). In the normal uterus, the analysis of immunoreaction against c-erbB-2 was assessed independently in the surface epithelium (SE), the superficial and deep glandular epithelia (SGE and DGE, respectively) and stromal compartment, using the same scoring system.

Statistical analysis

Statistical analysis was performed using chi-square and Fisher exact tests in the IBM SPSS STATISTICS BASE 20.0 software®. p Values < 0.05 were regarded as statistically [6] significant.

Results

Animals

The 34 FEA used in this study were diagnosed in queens with an age range of 1–15 years (mean 7.8 years); this information relates to 26 queens, since this information was not declared for the remaining eight animals. Breeds included Domestic Shorthaired cats (n = 24; 70.6%), Siamese (n = 2; 5.9%) and Persian (n = 1; 42.9%); in seven cases, breed was omitted. Contraception was confirmed in nine (26.5%) animals, whilst it was denied in 3 (8.8%) cases. In the remaining 22 cases, data concerning contraception was not mentioned in the form. When such information existed, the length of the treatment was not declared. The reasons leading to the request of histopathological analyses included clinical signs of uterine disease (n = 14; 41.2%), concurrent mammary tumour (n = 10; 29.4%) and detection of uterine lesions during routine ovariohysterectomy (n = 4; 11.8%).

Normal uterine samples (n = 23) used as controls were obtained from post-pubertal animals submitted to routine ovariohysterectomy. Queens' age was declared for 16 controls and ranged between 7 months and 8 years of age (mean 1.4 years). Breeds included Domestic Shorthaired cats (n = 7; 30.4%) and Persian (n = 1; 4.3%); this information was not specified for the remaining animals. In controls, none of the queens were submitted to contraceptive treatment.

Histopathological features

Feline endometrial adenocarcinomas (FEA) morphology was characterized by papillary proliferation, lined by multiple layers of neoplastic cells supported by a fibrovascular stroma. Other architectural patterns scarcely present within the tumours included solid, tubular and glomeruloid patterns. These features supported the classification of the tumours as a papillary serous type (Mutter and Crum 2000; Saraiva et al. 2012). Neoplastic cells were pleomorphic, mostly columnar shaped, with a moderate amount of eosinophilic cytoplasm and round-to-oval, vesicular or hyperchromatic nuclei that lost the normal polarity. Numerous multinucleated cells were present within and at the surface of the lesions. A variable number of clear cells was also observed. Nucleoli were evident and occasionally intranuclear clear inclusions were also found. Randomly distributed areas of necrosis within the tumours were frequently present. Histopathological features presumably related to tumour dedifferentiation are summarized in Table 1. Seventeen tumours (50.0%) had a low to moderate degree of atypia, whereas the other half of the sample (n = 17; 50.0%) was characterized by high atypia. The majority of tumours (n = 24; 70.6%) showed a mean number of mitoses per HPF between 1 and 5, while in 7 tumours (20.6%) it was <1 and in a minority of cases (n = 3; 8.8%) it was >5 mitoses. In normal samples, the surface epithelium in either FS or LS consistently presented <1 mitosis per

HPF (n = 12 and n = 11, respectively; 100.0%). The mean number of mitoses per HPF in tumours was higher, compared to the normal epithelia (p ≤ 0.0001) (Table 1). In the FS, the glandular epithelia (both the SGE and DGE) showed <1 mitosis per HPF. In the LS, the mean number of mitoses differed between the SGE and the DGE, though without statistical meaning: the SGE regularly presented a mean number of mitoses <1 (90.9%; n = 10) with only 1 sample showing a mean number of mitoses between 1 and 5 (9.1%), whilst the DGE showed a mean number of mitoses either <1 (n = 7; 63.6%) or between 1 and 5 mitoses (n = 4; 36.4%). Myometrial invasion was observed in 22 tumours (64.7%). Vascular and serosa impairment were present in 3 (8.8%) and 1 (2.9%) tumours, respectively. These cases occurred independently but they always co-existing with myometrial invasion.

c-erbB-2 immunohistochemistry

The immunoreaction against c-erbB-2 oncoprotein in FEA is summarized in Table 2. Of the 34 FEA cases, 14 (41.2%) were considered negative to c-erbB-2: four cases were classified as IHC0 and 10 cases as IHC1+ (11.8% and 29.4%, respectively). The remainder 20 FEA samples (58.8%) were positive for c-erbB-2

Table 2. Immunohistochemical expression of c-erbB-2 oncoprotein in FEA and normal endometrium. Samples were classified according to the score: IHC3+, circumferential membrane staining that is complete, intense and within >10% of tumour cells ('chicken-wire pattern'); IHC2+, circumferential membrane staining that is incomplete and or weak/moderate and within >10% of tumour cells or complete and circumferential membrane staining that is intense and within ≤10% of tumour cells; IHC1+, incomplete membrane staining that is faint/barely perceptible and within >10% of tumour cells; ICH0, no staining is observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤10% of tumour cells. Scores of IHC0 and IHC1+ were considered negative, whereas scores of IHC2+ and IHC3+ were considered positive and indicative of c-erbB-2 oncoprotein overexpression

	c-erbB-2 IHC score n (%)					
	Negative			Positive		
	IHC0	IHC1+	Total	IHC2+	IHC3+	Total
Follicular Stage (n = 12)[a,b]						
SE	1 (8.3)	0	1 (8.3)	9 (75.0)	2 (16.7)	11 (91.7)
SGE	0	0	0	4 (33.3)	8 (66.7)	12 (100.0)
DGE[c]	0	0	0	7 (58.3)	5 (41.7)	12 (100.0)
Luteal Stage (n = 11)[a]						
SE	0	1 (9.1)	1 (9.1%)	6 (54.5)	4 (36.4)	10 (90.9)
SGE[d]	0	0	0	9 (81.8)	2 (18.2)	11 (100.0)
DGE[c,d]	2 (18.2)	4 (36.4)	6 (54.6)	5 (45.4)	0	5 (45.4)
FEA (n = 34)[b]	4 (11.8)	10 (29.4)	14 (41.2)	18 (52.9)	2 (5.9)	20 (58.8)

IHC, immunohistochemical; FEA, feline endometrial adenocarcinomas; SE, surface epithelium; SGE, superficial glandular epithelium; DGE, deep glandular epithelium.
Different superscripts indicate a statistical significance: [a]P = 0.016; [b]P < 0.0001; [c]P = 0.004; [d]P = 0.024.

Table 1. Major histopathological features assessed in FEA cases

Histopathological features	Number of FEA in 34 (%)
Nuclear atypia	
Low to moderate	17 (50.0)
High	17 (50.0)
Mean number of mitoses per HPF	
<1	7 (20.6)
1–5	24 (70.6)
>5	3 (8.8)
Myometrium invasion	
Yes	22 (54.7)
No	12 (35.3)
Serosa invasion	
Yes	1 (2.9)
No	33 (97.1)
Vascular invasion	
Yes	3 (8.8)
No	31 (91.2)

FEA, feline endometrial adenocarcinomas; HPF, high power field.

(Fig. 1), being 18 (52.9%) scored as IHC2+ and 2 (5.9%) as IHC3+. The immunoreaction of c-erbB-2 in both normal and FEA stroma was consistently negative.

The stage of the cycle influenced the immunoreaction against c-erbB-2 in the normal uterus. Concerning the overall epithelial expression of c-erbB-2 in follicular and luteal stages, samples in FS were most commonly positive for c-erbB-2 (p = 0.016). In FS, the endometrial epithelia were generally positive for c-erbB-2 (Table 2; Fig. 2), with only 1 case (8.3%) being negative for this molecule in the SE. Scores varied between IHC2+ and IHC3+, accounting 75.0% (n = 9) and 16.7% (n = 2) in the SE, 33.3% (n = 4) and 66.7% (n = 8) in the SGE, and 58.3% (n = 7) and 41.7% (n = 5) in the DGE, respectively. In LS, the SE was negative in only 1 sample (9.1%), being positive in 10 specimens (90.9%), represented by six samples (54.5%) with a score IHC2+ and 4 (36.4%) with IHC3+. While the SGE in LS was considered positive in all the cases, being 9 (81.8%) scored as IHC2+ and 2 (18.2%) scored as IHC3+, the DGE was mainly negative (n = 6; 54.5%); 2 cases (18.2%) were scored as IHC0 and 4 (36.4%) samples had a score of IHC1+. Only five samples (45.4%) were found positive for c-erbB-2 with a score of IHC2+. In LS, the SGE was more frequently positive for c-erbB-2 than the DGE (p = 0.024). Positivity for c-erbB-2 was more frequent in DGE of the FS than in the homologous counterpart of the LS (p = 0.004). FEA were more likely negative for c-erbB-2 expression than controls of FS (p ≤ 0.0001). No significant differences were detected between FEA and LS.

In tumours, c-erbB-2 expression was compared with nuclear atypia, number of mitoses per HPF and myometrium, serosa and vascular invasion, all features presumably indicative of tumour dedifferentiation. However, no statistical differences were recorded.

Discussion

The present study proposed to establish the pattern of immunohistochemical expression for c-erbB-2 oncoprotein in FEA, compared to the normal feline endometrium and to explore putative associations between c-erbB-2 and the histopathological features of FEA that might be related to poor prognosis and worse outcome. To the best of our knowledge this is the first description of c-erbB-2 immunohistochemical expression in feline neoplastic endometrium.

Despite the rarity of endometrial adenocarcinomas in domestic species except cows and the rabbits (Cotchin 1964; McEntee and Nielsen 1976; Kennedy et al. 1998), the increasing number of reports on FEA questions the possibility that these tumours may be more
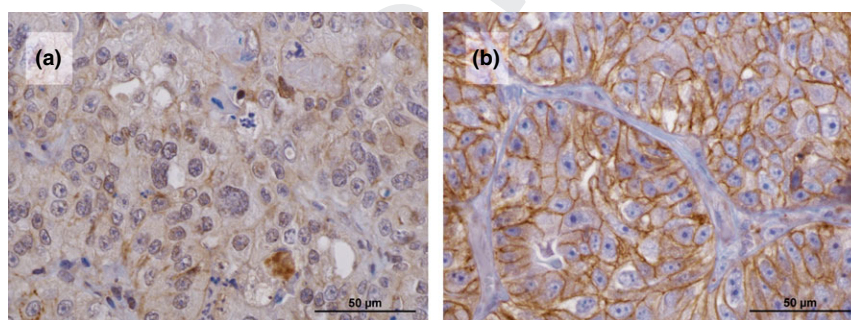

Fig. 1. Immunohistochemical expression of c-erbB-2 in feline endometrial adenocarcinomas (FEA), counterstained with Mayer's haematoxylin. Bar = 50 μm. (a) A negative scoring, with cells showing incomplete membrane staining that is faint/barely perceptible and within >10% of tumour cells, scored as IHC1+; (b) Positive FEA with cells displaying strong and complete membrane staining (referred as *chicken-wire pattern*) scored as IHC3+
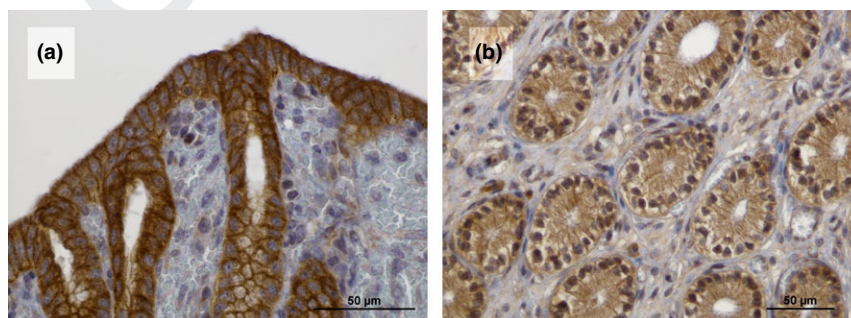

Fig. 2. Immunohistochemical expression of c-erbB-2 in the normal feline endometrium, counterstained with Mayer's haematoxylin. Bar = 50 μm. (a) In the follicular stage, the superficial epithelium shows positivity for c-erbB-2. (b) In the luteal stage, cells in the deep glandular epithelium are negative for c-erbB-2 immunostaining

common than the literature refers (Gil da Costa et al. 2009; Anderson and Pratschke 2011; Cho et al. 2011; Payan-Carreira et al. 2013; Sontas et al. 2013). Therefore, additional studies addressing FEA characterization are required.

The 34 cases used herein were classified as FEA of the papillary serous type, using previously reported criteria (Preiser 1964; Belter et al. 1968; McEntee and Nielsen 1976; Martín de las Mulas et al. 1995; Miller et al. 2003; Gil da Costa et al. 2009; Anderson and Pratschke 2011; Saraiva et al. 2012, 2015a). Moreover, there were also evaluated for specific histological features of malignancy (Miller et al. 2003; Horn et al. 2007; Goldschmidt et al. 2011), namely nuclear atypia, mitotic activity and myometrial, vascular and/or serosa invasion.

The c-erbB-2 molecule was expressed in 58.8% of the cases under study. In women, c-erbB-2 positive phenotype has been associated with high-grade tumours (Ferrandina et al. 2005; Mariani et al. 2005) and to early stages of the disease (Sugimoto et al. 2007). Data gathered in the present study failed to evidence an association between c-erbB-2 scoring and histopathological malignant features mentioned above, as it has been also referred regarding the women endometrial carcinomas (Ferrandina et al. 2005; Togami et al. 2012). Therefore, it can be speculated that c-erbB-2 expression might not have a great impact in the progression or the prognosis of FEA.

In women endometrial carcinomas, myometrial invasion is correlated with proliferation, risk of recurrence and poor outcome (Prat 2004). Mitotic indexes are used in routine histopathological diagnosis to assess proliferation and are often a predictor of malignancy. Neoplastic cells are often compared to trophoblastic cells on respect to invasiveness. However, studies in humans showed that the cells of the cyto- and the syncytiotrophoblast seldom express c-erbB-2 protein (Wang et al. 1992; Mühlhauser et al. 1993), suggesting that this molecule may not be directly associated with proliferation events, but mainly with differentiation phenomena. Thus, a positive expression of c-erbB-2 oncoprotein seems unrelated to poor clinical outcome of FEA. However, to assure this hypothesis, additional studies in follow-up of FEA and in feline placenta are needed to further investigate the potential prognostic value of FEA histological features of malignancy.

The present study also localized the c-erbB-2 protein in the normal feline uterus in the FS and LS of the cat oestrous cycle. Differences in the expression of c-erbB-2 between the normal feline endometrium and in endometrial hyperplasia had been determined (Misirlioglu et al. 2006). In that study, it was referred that the c-erbB-2 content in SE of normal feline uterus was lower than that of the glandular epithelium, contrasting with the findings in the present study. The differences may be explained by the fact that a different antibody or scoring systems were used, as well as by the fact that the oestrous cycle was not staged, therefore impairing any inference on eventual cyclic changes in this molecule expression. Still, the results reported herein showed that c-erbB-2 expression was confined to the epithelial compartment, as it has also been reported for human endometrium (Wang et al. 1992). Interestingly, in the present study, the expression of c-erbB-2 was significantly higher in follicular stage of feline endometrium compared to the luteal stage. Van Dam et al. (1991) [7] detected non-significantly higher levels of c-erbB-2 oncoprotein in the proliferative phase of pre-menopausal endometrium (grossly equivalent to the FS described herein), compared with the secretory phase (which would correspond to the LS in our work), alike to the results presented herein.

Oestrogens increase the levels of epidermal growth receptor (EGFR) in the endometrium (Mukku and Stancel 1985), and the cyclic interchange between oestrogens and progesterone has been proved to have a greater effect on EGF synthesis (Imai et al. 1995). Steroids also determine the interplay between the epithelial and stromal elements of the endometrium, which seems to have a major role in the endometrial proliferation and differentiation (Pierro et al. 2001). Once c-erbB-2 shares functional and morphological homologies with EGFRs, it could mediate oestrogen-induced proliferation (Maia et al. 2002). However, in a previous report it was showed that ER-α is underexpressed in FEA compared to normal endometrium, suggesting that the proliferation of FEA might be controlled by a different pathway involving local growth factors, rather than by ER-α expression (Saraiva et al. 2015b). It was also evidenced that the proliferative indexes, estimated on the basis of Ki-67 (MIB-1) immunolabelling, were higher in FEA than in the normal endometrium, irrespectively of the oestrous stage considered (Saraiva et al. 2015b). However, a positive correlation between c-erbB-2 and MIB-1 was described in human endometrial carcinomas (Ioachim et al. 2002), which seems to contrast with results reported herein. In fact, c-erbB-2 expression in LS was higher in the SGE compared to DGE. However, the persistency of proliferation of the basal glands during LS, which is reflected by the increased coiling reported in this stage (Chatdarong et al. 2005), should accompany an increase in c-erbB-2 expression if this pathway was involved in epithelial proliferation of feline endometrium. Watson et al. (1996) proposed that [8] differentiation and decidualization in the LS is a progesterone-mediated event, regulated by EGF. Payan-Carreira et al. (2014) also suggested that progesterone is an important driver for differentiation events occurring in the canine endometrium during the LS. All the above agreed with the involvement of c-erbB-2 in differentiation phenomena occurring during trophoblast invasion, as suggested by Mühlhauser et al. (1993).

These could explain the absence of association between the c-erbB-2 protein expression and the

histopathological malignant features expressed in FEA, such as the mitotic index or the invasiveness. Therefore, data gathered in the current study regarding c-erbB-2 molecule sustain the hypothesis raised in previous reports that other pathways controlling epithelial proliferation in the feline endometrium may be involved in FEA carcinogenesis (Saraiva et al. 2015b). Therefore, further studies ought to address the importance of the loop between oncogenes, growth factors and steroid hormones in the determination of proliferative and differentiation phenomena in feline cyclic and diseased endometrium.

Concluding, the c-erbB-2 immunohistochemical location and pattern of distribution in FEA and the normal endometrium in FS and LS is described herein for the first time. The majority of the FEA cases under study were positive to c-erbB-2 immunolabelling; however, a decrease in the expression of this protein in FEA was observed when compared to the FS of normal endometrium. No associations were established between c-erbB-2 expression and the histopathological features potentially related to FEA dedifferentiation and poor outcome. The expression of c-erbB-2 was significantly higher in the epithelia of the FS compared to LS, which suggests that this protein is influenced by functional changes occurring during the feline oestrous cycle. Additional studies are required to further investigate the role of c-erbB-2 in feline endometrial physiology and carcinogenesis.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

ALS, RP-C, FG and MAP were involved in the acquisition of clinical and histopathological data, data analysis and interpretation, as well as the manuscript writing and the reviewing of the literature. MAP and ALS were responsible for the immunohistochemical analysis. FF and LML were responsible for technical assistance. ALS, RP-C, FG and MAP contributed to the manuscript writing and the reviewing of the literature. All authors read and approved the final manuscript.

References

Anderson C, Pratschke K, 2011: Uterine adenocarcinoma with abdominal metastases in an ovariohysterectomised cat. J Feline Med Surg **13**, 44–47.

Belter LF, Crawford EM, Bates HR, 1968: Endometrial adenocarcinoma in a cat. Pathol Vet **5**, 429–431.

Brix DM, Clemmensen KK, Kallunki T, 2014: When good turns bad: regulation of invasion and metastasis by ErbB2 receptor tyrosine kinase. Cells **3**, 53–78.

Buza N, Roque DM, Santin AD, 2014: HER2/neu in endometrial cancer: a promising therapeutic target with diagnostic challenges. Arch Pathol Lab Med **138**, 343–350.

Chatdarong K, Rungsipipat A, Axnér E, Linde Forsber C, 2005: Hysterographic appearance and uterine histology at different stages of the reproductive cycle and after progestagen treatment in the domestic cat. Theriogenology **64**, 12–29.

Cho S-J, Lee H-A, Hong S, Kim O, 2011: Uterine adenocarcinoma with feline leukaemia virus infection. Lab Anim Res **27**, 347–351.

Cotchin E, 1964: Spontaneous uterine cancer in animals. Br J Cancer, **XVIII**, 209–227.

Espinosa de los Monteros A, Fernández A, Millán MY, Rodríguez F, Herráez P, Martín de las Mulas J, 1999: Coordinate expression of cytokeratins 7 and 20 in feline and canine carcinomas. Vet Pathol **36**, 179–190.

Ferrandina G, Ranelletti FO, Gallota V, Martinelli E, Zannoni GF, Gessi M, Scambia G, 2005: Expression of cyclooxygenase-2 (COX-2), receptors for estrogen (ER), and progesterone (PR), p53, ki67, and neu protein in endometrial cancer. Gynecol Oncol **98**, 383–389.

Gil da Costa RM, Santos M, Amorim I, Lopes C, Dias Pereira P, Faustino AM, 2009: An immunohistochemical study of feline endometrial adenocarcinoma. J Comp Pathol **140**, 254–259.

Goldschmidt M, Peña L, Rasotto R, Zappulli V, 2011: Classification and grading of canine mammary tumors. Vet Pathol **48**, 117–131.

Grushko TA, Filiaci VL, Mundt AJ, Ridderstråle K, Olopade OI, Fleming GF, 2008: An exploratory analysis of HER-2 amplification and overexpression in advanced endometrial carcinoma: a gynecologic oncology group study. Gynecol Oncol **108**, 3–9.

Horn L-C, Meinel A, Handzel R, Einenkel J, 2007: Histopathology of endometrial hyperplasia and endometrial carcinoma – an update. Ann Diagn Pathol **11**, 297–311.

Imai T, Kurachi H, Adachi K, Adachi H, Yoshimoto Y, Homma H, Tadokoro C, Takeda S, Yamaguchi M, Sakata M, Sakoyama Y, Miyake A, 1995: Changes in epidermal growth factor receptor and the levels of its ligands during menstrual cycle in human endometrium. Biol Reprod **52**, 928–938.

Ioachim EE, Goussia AC, Kitsiou EG, Charalabopoulos K, Mermiga E, Stefanaki S, 2002: Immunohistochemical expression of Retinoblastoma gene product in normal, hyperplastic and malignant endometrium. Correlation with p53 protein expression, c-erbB-2, hormone receptors' status and proliferative activity. Dis Markers **18**, 143–152.

Kennedy PC, Cullen JM, Edwards JM, Goldshmidt MH, Larsen S, Munson L, Nielsen S, 1998: Histological Classification of Tumors of the Genital System of Domestic Animals. Armed Forces Institute of Pathology in cooperation with the American Registry of Pathology and The World Health Organization Collaborating Center for Worldwide Reference on Comparative Oncology, Washington, DC, pp. 32.

Maia H Jr, Maltez A, Athayde C, Coutinho EM, 2002: C-erbB-2 over-expression in endometrial hyperplasia induced by estrogens. Maturitas **43**, 41–47.

Mariani A, Sebo TJ, Katzmann JA, Riehle DL, Dowdy SC, Keeney GL, Lesnick TG, Podratz KC, 2005: HER-2/neu overexpression and hormone dependency in endometrial cancer: analysis of cohort and review of literature. Anticancer Res **25**, 2921–2927.

Martín de las Mulas J, Espinosa de los Monteros A, Carrasco L, van Niel M, Fernández A, 1995: Immunohistochemical distribution pattern of intermediate

filament proteins in 50 feline neoplasms. Vet Pathol **32**, 692–701.

McEntee K, Nielsen SW, 1976: XVI. Tumours of the female genital tract. Bull World Health Organ **53**, 217–226.

Millanta F, Calandrella M, Citi S, Della Santa D, Poli A, 2005: Overexpression of HER-2 in feline invasive mammary carcinomas: an immunohistochemical survey and evaluation of its prognostic potential. Vet Pathol **42**, 30–34.

Miller MA, Ramos-Vara JA, Dickerson MF, Johnson GC, Pace LW, Kreeger JM, Turnquist SE, Turk JM, 2003: Uterine neoplasia in 13 cats. J Vet Diagn Invest **15**, 575–522.

Misirlioglu D, Nak D, Sevimli A, Nak Y, Ozyigit MO, Akkoc A, Cangul IT, 2006: Steroid receptor expression and HER-2/neu (c-erbB-2) oncoprotein in the uterus of cats with cystic endometrial hyperplasia-pyometra complex. J Feline Med Surg **53**, 225–229.

Mühlhauser J, Crescimanno C, Kaufmann P, Höfler H, Zaccheo D, Castellucci M, 1993: Differentiation and proliferation patterns in human trophoblast revealed by c-erbB-2 oncogene product and EGF-R. J Histochem Cytochem **41**, 165–173.

Mukku VR, Stancel GM, 1985: Regulation of epidermal growth factor receptor by estrogen. J Biol Chem **260**, 9820–9824.

Mutter GL, Crum CP, 2000: Tumors of the endometrium. In: Fletcher CDM (ed.), Diagnostic Histopathology of Tumors, 2nd edn. Churchill Livingstone, London, pp. 648–667.

Ordás J, Millán Y, Dios R, Reymundo C, Martín de las Mulas M, 2007: Protooncogene HER-2 in normal, dysplastic and tumorous feline mammary glands: an immunohistochemical and chromogenic in situ hybridization study. BMC Cancer **7**, 179.

Papparella S, Roperto F, 1984: Spontaneous uterine tumors in three cats. Vet Pathol **21**, 257–258.

Payan-Carreira R, Saraiva A, Santos T, Vilhena H, Sousa A, Santos C, Pires M, 2013: Feline endometrial adenocarcinoma in females < 1 year old: a description of four cases. Reprod Domest Anim **48**, e70–e77.

Payan-Carreira R, Santos C, Miranda S, Pereira RM, Santos D, Pires MA, 2014: Temporal changes in neutral endopeptidase/CD10 immunoexpression in the cyclic and early pregnant canine endometrium. Theriogenology **82**, 815–826.

Peña L, Gama A, Goldschmidt MH, Abadie J, Benazzi C, Castagnaro M, Díez L, Gärtner F, Hellmén E, Kiupel M, Millán Y, Miller MA, Nguyen F, Poli A, Sarli G, Zappulli V, Martín de las Mulas J, 2014: Canine mammary tumors: a review and consensus of standard guidelines on epithelial and myoepithelial phenotype markers, HER2, and hormone receptor assessment using immunohistochemistry. Vet Pathol **51**, 127–145.

Pierro E, Minici F, Alesiani O, Miceli F, Proto C, Screpanti I, Mancuso S, Lanzone A, 2001: Stromal-epithelial interactions modulate estrogen responsiveness in normal human endometrium. Biol Reprod **64**, 831–838.

Prat J, 2004: Prognostic parameters of endometrial carcinoma. Hum Pathol **35**, 649–662.

Preiser H, 1964: Endometrial adenocarcinoma in a cat. Pathol Vet **1**, 485–490.

Rasotto R, Caliari D, Castagnaro M, Zanetti R, Zappulli V, 2011: An immunohistochemical study of HER-2 expression in feline mammary tumours. J Comp Pathol **144**, 170–179.

Saraiva AL, Payan-Carreira R, Gärtner F, Pires MA, 2012: Feline endometrial adenocarcinomas. In: Longoria MA, Alcalá JI (eds), Adenocarcinoma: Pathogenesis Treatment and Prognosis. Nova Science Publishers, Hauppauge, NY, pp. 175–189.

Saraiva AL, Payan-Carreira R, Gärtner F, Santana I, Rêma A, Lourenço LM, Pires MA, 2015a: Immunohistochemical expression of cyclooxygenase-2 (COX-2) in feline endometrial adenocarcinoma and in normal and hyperplastic endometria. Reprod Domest Anim **50**, 333–340.

Saraiva AL, Payan-Carreira R, Gärtner F, Fortuna da Cunha M, Rêma A, Faria F, Lourenço LM, Pires MA, 2015b: An immunohistochemical study on the expression of sex steroid receptors, Ki-67 and cytokeratins 7 and 20 in feline endometrial adenocarcinomas. BMC Vet Res **11**, 204.

Sontas BH, Erdogan Ö, Apaydin Enginler SÖ, Turna Yilmaz Ö, Şennazli G, Ekici H, 2013: Endometrial adenocarcinoma in two young queens. J Small Anim Pract **54**, 156–159.

Sugimoto T, Koizumi T, Sudo T, Yamaguchi S, Kojima A, Kumagai S, Nishimura T, 2007: Correlative expression of cyclooxygenase-1 (Cox-1) and human epidermal growth factor receptor type-2 (Her-2) in endometrial cancer. Kobe J Med Sci **53**, 177–187.

Togami S, Sasajima Y, Oi T, Ishikawa M, Onda T, Ikeda S-I, Kato T, Tsuda H, Kasamatsu T, 2012: Clinicopathological and prognostic impact of human epidermal growth factor receptor type 2 (HER2) and hormone receptor expression in uterine papillary serous carcinoma. Cancer Sci **103**, 926–932.

Van Dam PA, Lowe DG, Watson JV, James M, Chard T, Hudson CN, Sheperd JH, 1991: Multiparameter flow-cytometric quantification of epidermal growth factor receptor and c-erbB-2 oncoprotein in normal and neoplastic tissues of the female genital tract. Gynecol Oncol **42**, 256–264.

Wang DP, Fujii S, Konishi I, Nanbu Y, Iwai T, Nonogaki H, Mori T, 1992: Expression of c-erbB-2 protein and epidermal growth factor receptor in normal tissues of the female genital tract and in the placenta. Virchows Arch A Pathol Anat Histopathol **420**, 385–393.

Watson H, Franks S, Bonney RC, 1996: Regulation of epidermal growth factor receptor synthesis by ovarian steroids in human endometrial cells in culture. J Reprod Fertil **107**, 199–205.

Winston J, Craft DM, Scase TJ, Bergman PJ, 2005: Immunohistochemical detection of HER-2/neu expression in spontaneous feline mammary tumours. Vet Comp Oncol **3**, 8–15.

Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, Fitzgibbons P, Hanna W, Jenkins RB, Mangu PB, Paik S, Perez EA, Press MF, Spears PA, Vance GH, Viale G, Hayes DF, American Society of Clinical Oncology, College of American Pathologists, 2013: Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol **31**, 3997–4013.

Yarden Y, Sliwkowski MX, 2001: Untangling the ErbB signaling network. Nat Rev Mol Cell Biol **2**, 127–137.

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