Activation of AKT2 in feline mammary carcinoma: a new prognostic factor in feline mammary tumors

Activation of AKT in feline mammary carcinoma: A new prognostic factor for feline mammary tumours

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A B S T R A C T

The PI3K/AKT/PTEN pathway is involved in the pathogenesis of several human cancers. This study investigated the biological and prognostic value of PI3K/AKT/PTEN pathway dysregulation in feline mammary tumours. Expression of p-AKT, HER2, PTEN and steroid receptors was assessed by immunohistochemistry (IHC) in 27 malignant and 12 benign mammary tumours from 39 female cats followed up over a 24-month period. Feline mammary carcinoma (FMC) cell lines were analyzed by Western blot and the feline AKT gene sequence was characterized.

p-AKT expression statistically correlated with tumour malignancy, histological dedifferentiation and clinical recurrence. The animals with tumours expressing p-AKT had a shorter disease-free period than those with p-AKT-negative tumours. AKT activation was associated with HER2 expression and PTEN down-regulation, as occurs in human breast cancer, and feline AKT sequencing showed high homology with the human AKT gene. No AKT activation was observed in relation to either oestrogen receptor a (ERa) or progesterone receptor expression. Taken together, these data offer an explanation for AKT signalling and its role in FMC pathogenesis and prognosis, shedding new light on similarities between feline mammary tumours and hormone-independent breast cancer.

Introduction

Since 1980, feline mammary carcinoma (FMC) has been recognized as a suitable animal model for studying human breast cancer because it shares epidemiological, morphologic and prognostic features with human breast carcinoma (Misdorp and Weijer, 1980). FMCs are mostly oestrogen receptor alpha (ERa) negative and progesterone receptor (PR) positive, although PR levels have been shown to decrease in invasive carcinomas (Millanta et al., 2006; Martin de las Mulas et al., 2002) suggesting loss of steroid dependence during malignant progression (Hamilton et al., 1976; Lana et al., 2007; Rutteman et al.,1991). FMC is therefore considered a model for hormone-independent human breast carcinomas (Martin et al., 1984; Martin de las Mulas et al., 2000a,b, 2002).

In order to find useful prognostic and therapeutic applications, a number of human oncogenes and receptors have been investigated in FMC (Hahn et al., 1994), including HER2/neu (De Maria et al., 2005; Ordas et al., 2007), feline RON/stk (De Maria et al., 2002), phosphatase and tensin homolog (PTEN) (Ressel et al., 2009),steroid receptors (Martin de las Mulas et al., 2002) and STAT3 (Petterino et al., 2007).

AKT, also known as protein kinase B, is a serine/threonine protein kinase which, when activated by phosphorylation (p-AKT), promotes growth factor-mediated cell growth, proliferation, migration and survival (Tokunaga et al., 2006). AKT plays a decisive role in cell survival by direct phosphorylation of the transcription factors controlling anti-apoptotic gene expression (Nicholson and Anderson, 2002; Perez-Tenorio et al., 2002; Tokunaga et al., 2008).

AKT is activated by a variety of tyrosine kinase receptors (RTKs), including EGFR1 and EGFR2/HER2, in a phosphoinositide-3-OH kinase (PI3K)-dependent manner (Nicholson and Anderson, 2002). Moreover, growth factors of the epidermal growth factor (EGF) family may utilize the PI3K/AKT pathway to activate ERa and confer hormone-independent growth to breast carcinoma (Stoica et al., 2003). Briefly, PI3K in its activated form can generate phosphatidylinositol 3,4,5-triphosphate (PIP3) which acts as a second messenger to activate AKT; under physiological conditions PIP3 levels are regulated by PTEN and other phosphatases (Perez-Tenorio et al., 2002). In many human cancers including breast carcinoma (Luo et al., 2003), aberrant activation of the PI3K-AKT pathway has been widely implicated as a driver of tumour development, progression and therapeutic resistance (Tokunaga et al., 2008).

In feline mammary tumours, HER2 is over-expressed in about 39-56.9% of cases (De Maria et al., 2005), where it is a negative prognostic factor (Millanta et al., 2005). Loss of PTEN has been reported in 76% of FMC cases, showing that female cats with PTEN-negative tumours had a worse prognosis (Ressel et al., 2009); however, there are no data about AKT in feline mammary tumours.

The aim of this study was (1) to explore the PI3K/AKT/PTEN pathway in FMC by analyzing AKT protein expression both in vivo and in vitro, thus confirming its value as a prognostic factor and (2) to characterize the molecular features of the feline AKT gene.

Materials and methods

Tissue samples and histology

Tissue samples from 39 feline mammary tumour cases treated between 2000 and 2008 at the Department of Animal Pathology, University of Turin, were examined. Archival data on age, breed, ovariectomy status, tumour size were retrieved from the hospital database. Postoperative clinical, radiological and echographic examinations at 6, 12, 18 and 24 months after surgery were performed by veterinarians to detect the presence of distant organ metastases or local recurrence of the primary tumour. Animals which had died due to mammary carcinoma were necropsied for confirmation of the pathological diagnosis; subjects which had died of non-tumour-related causes during the follow-up period were excluded from the study.

Samples were fixed in 4% neutral buffered formalin, paraffin embedded, sectioned at 4 μm and stained with haematoxylin and eosin. Tumours were classified according to the World Health Organization (WHO) classification for tumours of domestic animals (Misdorp et al., 1999). In addition, carcinomas were classified by differentiation status (Castagnaro et al., 1998).

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was carried out on 39 samples and five lymph node metastases using 4 µm sections of formalin-fixed, paraffin-embedded tissues. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections underwent high-temperature antigen unmasking by incubation at 98 °C with citric acid buffer (pH 6.0).

The primary anti-human antibodies included were against phospho-AKT$_{Ser}$473-736 (Ell rabbit monoclonal; Cell Signaling Technology, New England Biolabs; 1:50 dilution), c-erbB-2 oncoprotein (rabbit polyclonal anti-human, Dako; 1:200 dilution), PTEN (mouse monoclonal, clone A2B1, Santa Cruz Biotechnology; 1:50 dilution), PR (monoclonal, clone PR10A9 IgG2A, Immunotech Laboratories; 1:750 dilution) and ERot (rabbit polyclonal, Zymed Invitrogen; 1:80 dilution). Antibodies were detected with avidin-biotin peroxidase complex techniques using an Envision kit (Dako). Adequate external positive controls were used: paraffin-embedded sections of FMCm cells for p-AKT, human breast carcinoma for HER2, feline normal kidney for PTEN and feline normal uterus for ERot and PR. As negative controls, sections were incubated without the primary antibodies.

Immunolabelled slides were randomized and masked for blinded examination performed independently by three observers (LM, SI and YM) and, when there was disagreement (<5% of the slides), a consensus was obtained using a multi-head microscope. Cytoplasmic staining of p-AKT and PTEN was scored using a modified semiquantitative scoring system (range 0-7; positivity Se3), as described by Seow et al. (2010). HER2 immunoreactivity was scored according to the HerceptTest method (Dako) commonly used in human pathology and also adopted in FMCs (Ordas et al., 2007). Positivity for ERot and PR was evaluated using a semiquantitative scoring system (range 0-8; positivity Se3), as previously adopted for human mammary carcinomas (Allred et al., 1998).

Cell lines and Western blot analysis

Western blot (WB) analysis was carried out on six feline mammary tumour cell lines (P248m, FYCp, FKNp, FNNm, FMCp, FMCm) kindly supplied by Dr. Nobuo Sasaki (Takauji et al., 2007) and on FcLu corresponding to the feline lung normal cell line obtained from the American Type Culture Collection (ATCC). FYCp, FKNp, FNNm, FMCp, FMCm cells were grown in RPMI medium and P248m and FcLu cells in DMEM medium; both media were supplemented with 10% fetal calf serum, 100µg/mL penicillin, 100µg/mL streptomycin, 1.5 mg/mL fungizone (10µg/mL insulin were added to the medium of the P248 cell lines). Lysate deriving from MCF10 cell lines was used as p-AKT positive control.

Total proteins were extracted with boiling lysis buffer containing 1% SDS, 0.1 M Tris-HCl at pH 6.8. From each sample, 20 µg of total protein were separated on 8% SDS-polyacrylamide (PAGE) gels, transferred onto Hybond-C Extra membranes (Amersham Biosciences) and incubated overnight at 4 °C with the following primary antibodies: p-AKT (Cell Signaling Technology; 1:1000); c-erbB-2 (Dako;1:1000), PTEN (Santa Cruz Biotechnology; 1:200); AKT (Cell Signaling Technology; 1:1000). After washing in PBS-Tween (0.5%), the membranes were incubated with secondary horseradish peroxidase (HRP)-linked antibody diluted 1:1000 in PBS-Tween for 1 h. Finally, the membranes were washed in PBS-Tween for 30 min and incubated in enhanced chemiluminescence reagents (Super Signal West Pico Mouse IgG Detection Kit, Thermo Scientific).

Sequencing

To identify the feline AKT sequence, primers were designed on cDNA of the human AKT2 sequence (M95936), with the higher homology with canine and murine sequences corresponding to exons 9-13. Total RNA was extracted from the feline FcLu cell line with Trizol (Invitrogen) and reverse-transcribed in cDNA by IMPROMII reverse transcriptase (Promega). The obtained PCR products were purified from agarose gel using a NucleoSpin kit (Macherey-Nagel) and sequenced. Primer sequences are available in Supplementary Table SI.

Statistical analysis

The immunohistochemical results were analyzed using Fisher's exact test. The disease-free period was calculated using a log-rank test, $p<0.05$ was considered statistically significant.

Results

Epidemiologic and clinical data

The mean age of the animals was 10.4 ±3.77 years (range 6 months-20 years). Of the 39 animals, 9 (23%) were spayed and one was cyclically treated with progesterone. Twenty queens underwent simple nodulectomy with the tumours removed in toto; six underwent partial mastectomy with removal of the cranial and caudal healthy mammary glands, and 13 had a total mastectomy. At the time of diagnosis, histologically confirmed lymph node metastasis was present in five (22.7%) of the FMCs. No clinically or radiographically observable distant organ metastasis was present. Grossly, only five tumours were sg5 mm in diameter (Table 1); in one case (Case 6) the tumour measured 10 cm.

Histopathology

Histology showed 12 (30.8%) benign and 27 (69.2%) malignant tumours. The benign lesions included five lobular hyperplasia, three fibroadenomas and four adenomas. All 27 carcinomas (Table 1) were simple subtypes and classified as follows: six well differentiated carcinomas (WDCs); 15 moderately differentiated carcinomas (MDCs); and six poorly differentiated carcinomas (PDCs). Lymph node metastases were diagnosed in five cases: three arising from MDCs (Cases 16,17, 20) and two from PDCs (Cases 24, 25). Careful histological examination of selected carcinomas revealed metastatic invasion of the lymphatics or blood vessels in five (22.7%) and seven (31.82%) cases, respectively.

Follow-up

Of the 27 animals diagnosed with FMC, 12 did not present tumour recurrence and 15 relapsed between 3 and 18 months after surgery (Table 1). Of the 15 subjects with relapse, four presented local primary tumours and 11 presented distant organ metastases and died of cancer-related causes. In the animals diagnosed with malignancy, the mean disease-free period was 15.74 ± 8.22 months; all animals diagnosed with a benign tumour were still alive at the end of the study period and did not present lesion recurrence.

IHC analysis

p-AKT IHC staining was detected in both the cytoplasm and the nucleus, as mentioned in the antibody datasheet; however, as previously described in humans, staining localized at the nuclear level**Table 1**

Clinical data, histopathology and immunohistochemical findings.

Case no.	Age (years)	Ovariectomy	Surgery	Ø size (cm)	Grade	MI [a]	VI	Li	Relapse	IHC score p-AKT	IHC score HER2	IHC score PTEN	IHC score ERα	IHC score PR
1	12	No	Total	5	WDC	1 (3)	No	No	No	2	1	0	2	2
2	18	Yes	Nodulectomy	2.5	WDC	1 (3)	No	No	12 m	4	1	0	3	0
3	7	No	Nodulectomy	0.5	WDC	1 (5)	No	No	No	4	2	0	0	0
4	14	No	Total	1	WDC	1 (3)	No	No	No	2	1	0	4	4
5	6	Yes	Nodulectomy	3	WDC	2 (11)	No	No	No	2	1	0	0	0
6	10	No	Total	10	WDC	2 (17)	Yes	No	12 m	6	3	0	5	0
7	9	No	Nodulectomy	3	MDC	3 (21)	No	No	13 m	4	2	3	2	0
8	12	MAP	Total	4	MDC	3 (22)	No	No	12 m	2	0	0	0	0
9	9	No	Total	2	MDC	1 (8)	Yes	No	6 m	4	3	0	0	0
10	14	No	Nodulectomy	0.5	MDC	2 (12)	No	No	No	0	0	0	0	0
11	10	No	Partial	1.8	MDC	2 (14)	Yes	No	12 m	5	2	3	0	0
12	13	No	Partial	1	MDC	2 (11)	Yes	No	12 m	3	0	0	0	2
13	10	Yes	Partial	2	MDC	2 (15)	No	No	No	2	0	0	0	0
14	14	Yes	Total	1.5	MDC	2 (13)	No	No	18 m	4	1	0	0	0
15	9	Yes	Nodulectomy	0.5	MDC	2 (14)	No	No	No	5	0	0	2	3
16	8	No	Nodulectomy	1	MDC	3 (21)	No	Yes	3 m	6	0	0	0	2
17	13	No	Total	1	MDC	3 (32)	No	Yes	No	0	1	0	0	3
18	9	Yes	Total	2	MDC	3 (23)	No	No	3,5 m	3	2	0	0	0
19	14	Yes	Total	2	MDC	2 (17)	No	No	No	5	3	0	2	0
20	8	No	Partial	1.5	MDC	3 (32)	Yes	Yes	6 m	7	0	0	0	0
21	11	No	Total	0.5	MDC	3 (23)	No	No	No	2	0	0	2	0
22	12	No	Total	2	PDC	3 (27)	Yes	No	6 m	4	1	0	0	0
23	13	No	Nodulectomy	2	PDC	3 (24)	No	No	4 m	3	3	0	0	0
24	12	No	Total	2.5	PDC	3 (28)	No	Yes	12 m	4	0	0	0	0
25	12	No	Total	2	PDC	3 (24)	Yes	Yes	6 m	7	3	0	0	0
26	18	No	Partial	1	PDC	3 (28)	No	No	No	3	0	0	0	0
27	10	Yes	Partial	0.5	PDC	2 (15)	No	No	No	4	0	6	6	0

MI, mitotic index; VI, vascular invasion; Li, lymphatic invasion; MAP, periodically treated with progesterone; WDC, well differentiated carcinoma; MDC, moderately differentiated carcinoma; PDC, poorly differentiated carcinoma.

[a] mitotic count in 10 HPF (in brackets) and corresponding mitotic rate (1–3 points) is reported.

could be observed in the hyperplastic glands or adenomas, while cytoplasmic expression was associated with larger tumours and reduced disease-free survival. Accordingly, in this study, only cytoplasmic staining was considered (Shtilbans et al., 2008). In the samples with a high score of AKT expression, we found a uniform localization throughout the whole tumour. In those where AKT staining was irregular, it was localized in the tumour borders, particularly in the carcinomas. Focal distribution (<10% weak positiv-ity; total score = 2) was considered negative.

Cytoplasmic p-AKT expression was observed in 56.4% (22/39) of cases, HER2 membrane staining in 30.8% (12/39) and cytoplasmic PTEN staining in 17.9% (7/39). Nuclear immunoreactivity for ERa and PR was detected in 41% (16/39) and 17.9% (7/39) of lesions, respectively. All HER2-positive samples showed AKT activation ($p<0.0001$).

The immunohistochemical percentage of p-AKT, HER2, PTEN, ERa and PR expression in benign and malignant lesions is reported in Fig. 1. Weak cytoplasmic p-AKT expression was observed in 10% of epithelial tumour cells (score = 3) in 25% (3/12) of benign tumours, two of which were classified as fibroadenomas. Normal mammary glands did not express p-AKT. HER2 was detected in three cases (25%) with moderate incomplete membrane staining in at least 10% of tumour cells (HerceptTest: 2+) in one hyperplasia and two fibroadenomas. Cytoplasmic PTEN positivity was observed in normal mammary glands and in four benign lesions (33.33%). In all benign lesions (100%), positive oestrogen-responsive elements (Ere) were detected in the nuclei of epithelial cells occupying the luminal position, and PR was detected in the nuclei of suprabasal or basal cells of eight benign lesions (66.66%). Normal mammary glands were both ERa and PR positive.

Table 1 summarizes the results of IHC analysis of the 27 malignant lesions. p-AKT expression was noted in 67.67% (19/27) of FMCs (Fig. 2). A trend emerged between p-AKT activation and tumour dedifferentiation: 50% of WDCs, 67% of MDCs and 100% ofPDCs were positive. HER2 membrane staining was observed in 9/ 27 cases (33.33%). PTEN positivity was observed in 11% (3/27) of carcinomas (Case Nos. 7, 11, 27) and in the normal fibroblasts surrounding the tumours in 14/27 (51.85%) (Fig. 3). Nuclear ERe and PR immunodetection was present in four and three cases, respectively. All lymph node metastases were positive for p-AKT (Fig. 4) and HER2, and negative for ERa and PR.

p-AKT expression statistically correlated with tumour malignancy ($P<0.05$) and clinical recurrence ($p<0.05$). The animals with tumours expressing p-AKT had a shorter disease-free period compared to those with p-AKT-negative tumours (Fig. 5) (log-rank test $P = 0.001$). AKT was expressed in 60% of tumours with low mitotic index (MI = 1), in 70% of those with moderate MI (MI = 2) and in 75% of those with high MI (MI = 3); however, no statistical association was found between MI and p-AKT expression ($P > 0.05$ chi-square test).

Molecular studies

To further investigate the role of p-AKT in FMC, Western blot analysis against p-AKT, total AKT, PTEN and HER2 was performed on FMC cell lines (Fig. 6). A specific band of 60KD corresponding to human p-AKT was found in 5/6 FMC cell lines (Fig. 5). Total AKT signal was higher in the cells expressing low levels of p-AKT. HER2 was expressed in all FMC cell lines and in FcLu with a higher expression in FKNp, FMCm, P248m and FYCp. Total PTEN was expressed in all cell lines. The FMCm cells line showed higher expression of p-AKT and HER2 compared to cells derived from the primary tumour (FMCp).

We partially determined the sequence of feline AKT c-DNA from exon 9 to exon 13. The sequence was submitted to GenBank (accession number HM990969). Alignment with cDNA of human and canine sequences revealed an identity of 92%.

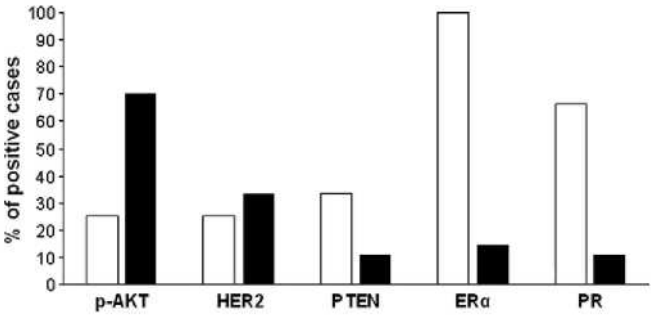

Fig. 1. Comparison of the expression of p-AKT, HER2, PTEN, ERot and PR between benign (white) and malignant (black) feline mammary tumours.

Discussion

Breast cancer is the most frequently diagnosed cancer in women. Feline mammary tumours may provide an excellent model of human mammary carcinomas for developing new therapeutic strategies, as demonstrated by molecular and histopathological studies (Misdorp and Weijer, 1980; Martin et al., 1984; Hahn et al., 1994; Munson and Moresco, 2007).

In this study we demonstrated that AKT activation was correlated with malignancy and tumour dedifferentiation in cats and that it was statistically associated with a short disease-free period. These results are in line with previous studies on breast cancer (Perez-Tenorio et al., 2002; Wu et al., 2008) and similar to an immunohistochemical study on 165 invasive breast cancers versus normal breast epithelium, fibroadenoma, intraductal hyperplasia and ductal carcinoma in situ (Zhou et al., 2004). The study found a progressive increase in detectable p-AKT from normal breast epithelium to hyperplasia and to tumour invasion, and that phosphorylation of AKT was associated with poor disease-free survival (Zhou et al., 2004).

Currently the most significant prognostic factors in FMC are tumour size, extent of surgery and histological grade. Recent studies have correlated poor prognosis with HER2 overexpression (Castagnaro et al., 1998; Viste et al., 2002; Millanta et al., 2005).

Because FMCs are highly aggressive and tend to metastasize rapidly to the lungs, not all veterinarians perform total mastectomy in such cases. Hayes and Mooney (1985) observed that radical mastectomy reduced local recurrence but did not increase overall survival time without adjuvant therapy (Novosad et al., 2006).

In our study, the FMCs were surgically removed by different techniques, which obtained tumour-free surgical margins but did not change the overall survival time. Early diagnosis and surgery remain the most effective methods to prevent recurrence. In this series, cases presenting with a tumour <0.5 cm in diameter did not relapse. We found a significant correlation between p-AKT expression and tumour recurrence and a significantly shorter disease-free period. These data suggest that, as in human cancers, AKT activation promotes cellular growth and proliferation in FMC (Sun et al., 2001; Perez-Tenorio et al., 2002; Wu et al., 2008; Seow et al., 2010).

HER2 overexpression was found in 33.33% of FMC, confirming previously published data (De Maria et al., 2005; Millanta et al., 2005), although Rasotto et al. (2010) reported HER2 overexpression in only 5.5% of FMC from 73 female cats. This disparity cannot be explained at present, and considering the importance of FMC as a model for human breast cancer, a standardized method for determination of HER2 expression and cellular localization in feline mammary tumours is needed.

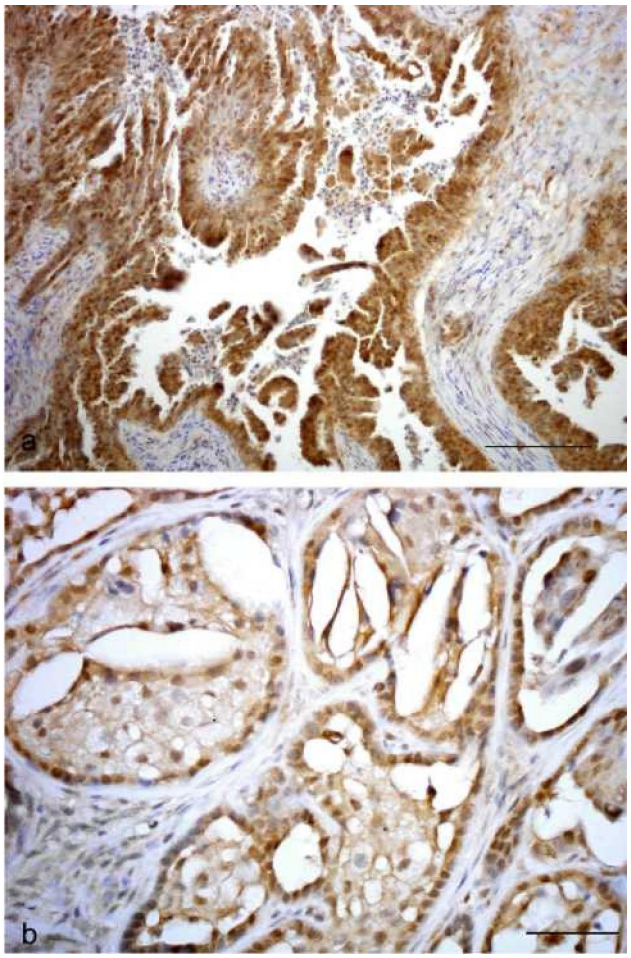

Fig. 2. Mammary gland, Case 25. Tubulopapillary carcinoma showing cytoplasmic staining for p-AKT (score 7) at lower (a) and higher (b) magnification. Streptavidin-biotin-peroxidase method. Mayer's hematoxylin counterstaining. Bar= 100 μm (a) and 50 μm (b).

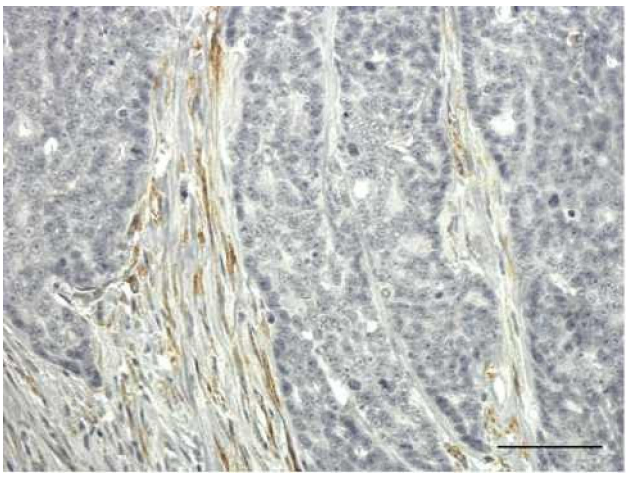

Fig. 3. Mammary gland, Case 21. Tubulopapillary carcinoma without immunore-activity for PTEN in cancer cells (score 0) but showing positive staining in surrounding stromal cells. Streptavidin-biotin-peroxidase method. Mayer's hematoxylin counterstaining. Bar = 200 μm.

We speculate that HER2 expression alone in benigntumours may be insufficient to induce a malignant phenotype and that further molecular alterations are necessary. In this study, IHC analysis demonstrated a positive association between HER2 expression and p-AKT activation, however, suggesting that HER2 is an upstream regulator of AKT signalling in FMC. Western blot analysis of FNNm and FMCp cell lines showed a low expression of HER2 and a high expression of p-AKT. These data suggest that AKT activation in these cells is not regulated by HER2 but by other molecular pathways, as demonstrated in human studies (Klinghof-fer et al., 1996; Duan et al., 1999; Okano et al., 2000). Further studies will help us better understand the other molecular pathways involved in AKT activation.

PTEN is a negative regulator of the AKT pathway. In a recent study on its role in FMC (Ressel et al., 2009), loss of PTEN expression as revealed by IHC was found in 76% of FMC and the female cats with PTEN-negative tumours had a worse prognosis. (Ressel et al., 2009). In our study, loss of PTEN expression was observed in 89% of FMCs, which could be related to the high rate of AKT activation in these cases, even if no statistical association was found between PTEN negative and p-AKT positive FMCs. Regarding the link between PTEN loss and HER2 expression in FMC, we hypothesize that the loss of PTEN, a negative regulator of AKT, and HER2 expression could promote AKT activation (p-AKT), so inducing proliferative and anti-apoptotic processes.

High PTEN expression in normal fibroblasts surrounding carcinomas was observed in this study. Recently, Trimboli et al. (2009) demonstrated that genetic inactivation of PTEN in stromal fibroblasts of the mouse mammary gland can accelerate the initiation, progression and malignant transformation of mammary epithelial tumours. Further experiments are needed to confirm this aspect in FMCs.

Hormone receptor status is an important parameter for predicting prognosis in human breast cancer, and feline mammary tumours are a model for studying ERa negative breast cancer (Martin et al., 1984; Martin de las Mulas et al., 2000a,b, 2002). While our data regarding ER expression in FMCs are consistent with the literature, we observed a lower percentage of PR expression in carcinomas compared to previous studies (Millanta et al., 2006; Martin de las Mulas et al., 2002). Since the number of spayed subjects cannot be considered and since we used the same technique than Martin de las Mulas et al. (2002), we assume that this disparity could be the consequence of tumour de-differentiation and acquired hormone independency (Millanta et al., 2006; Martin de las Mulas et al., 2000a,b, 2002). The different stage of oestrus between subjects could also have an influence. In human breast cancer, there is a positive correlation between p-AKT and both ER and PR status (Park and Kim, 2007). Our results demonstrate a significant, positive correlation between ERa expression and benign neoplasia, but no correlation between AKT activation and ERa or PR status. This difference suggests that AKT is probably not linked to hormone receptor status in FMC, strongly indicating the hormone independency of FMC.

The metastatic cell line (FMCm) showed a higher level of HER2 and p-AKT expression compared to the primary cell line (FMCp), suggesting that higher p-AKT and HER2 expression plays an important role in tumour progression. We found a low level of HER2 but a moderate activation of AKT in FNNm, suggesting that in this metastatic cell line AKT activation is not HER2-dependent and that other oncogenic events up-stream of AKT need to be investigated (Klinghoffer et al., 1996; Duan et al., 1999; Okano et al., 2000). Finally, not all the metastatic cell lines showed higher expression of p-AKT compared to the primary cell lines, demonstrating that tumour progression is not only p-AKT-dependent and that other molecular pathways are likely involved.

In humans, first-generation inhibitors of the PI3K/AKT/PTEN pathway have recently been shown to be effective in reducing tumour cell growth both in vitro and in vivo (Luo et al., 2003). Continuing efforts to develop specific, high-affinity inhibitors against the PI3K-AKT pathway may yield new therapeutics to treat human cancer. Spontaneous tumours in companion animals repre sent potential models to study innovative therapies for human cancer. Dogs and cats in particular can develop spontaneous tumours with histopathological and biologic behaviours comparable to specific human tumour subtypes. Specifically, feline mammary tumours share biological and molecular features with hormone-independent human breast cancer.

Previous studies have demonstrated that FMC is a model for aggressive, hormone-independent human breast cancer over-expressing HER2 (De Maria et al., 2005; Martin de las Mulas et al., 2000a,b). Our results demonstrate the role of AKT signalling in FMC pathogenesis and prognosis and shed new light on the similarities between hormone-independent breast cancer and FMC.

Conclusions

AKT plays a pivotal role in the pathogenesis of feline mammary tumours and its activation is a negative prognostic factor. The results provide further evidence that feline mammary tumour is a suitable model to interrogate new therapeutic strategies against breast cancer.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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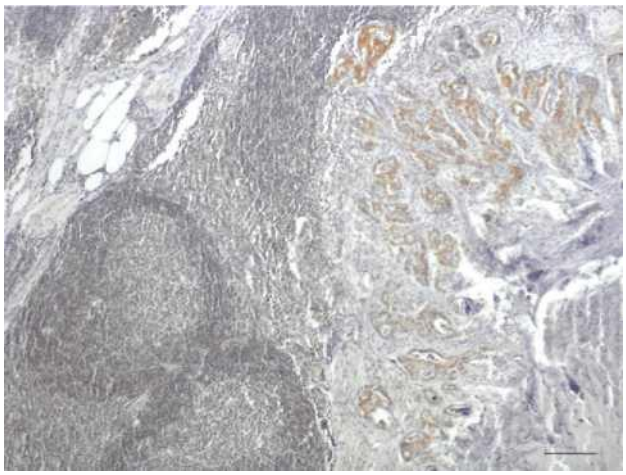

Fig. 4. Lymph node, Case 20. Lymph node metastasis of carcinoma with immuno-reactivity for p-AKT in cancer cells. Streptavidin-biotin-peroxidase method. Mayer's hematoxylin counterstaining. Bar = 100 μm.

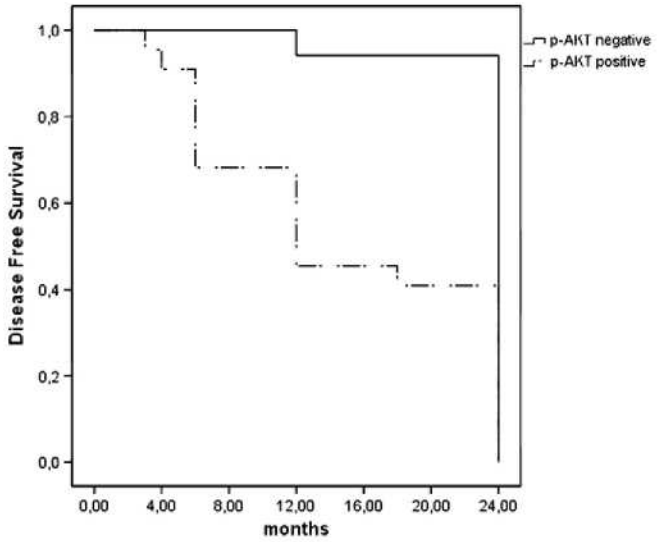

Fig. 5. Kaplan-Meier estimates for overall survival probability in p-AKT-positive and p-AKT-negative mammary carcinomas (P< 0.005).

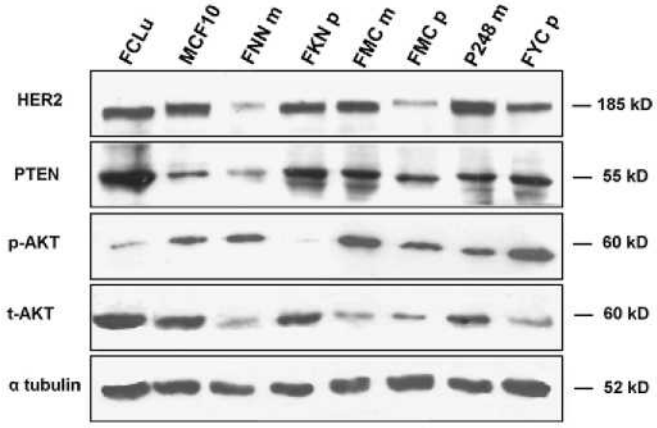

Fig. 6. Western blot analysis of HER2, PTEN, p-AKT and t-AKT expression in cell lines derived from feline mammary carcinoma (FMC) primary and metastatic lesions ('p' and 'm' denote cell lines derived from primary and metastatic lesions, respectively). MCF10 was used as a positive control for p-AKT. Tubulin expression was used as loading control.

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