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# Activation of Mammalian Target of Rapamycin in Canine Mammary Carcinomas: An Immunohistochemical Study

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## Summary

Mammalian target of rapamycin (mTOR) is a serine–threonine kinase involved in cell growth, proliferation and survival. Activation of mTOR has been reported in various tumour types, including human breast cancer; however, the expression of mTOR in canine mammary tumours has not been examined. In the present study, expression of the activated form of mTOR (phospho-mTOR [p-mTOR]) was examined immunohistochemically in five normal canine mammary glands, 45 canine mammary carcinomas and their corresponding metastatic lesions ($n = 15$). Phospho-mTOR was not expressed in normal canine mammary tissue, but cytoplasmic labelling was observed in 78% of canine mammary carcinomas. Two carcinomas had both cytoplasmic and nuclear labelling. No significant relationship was found between p-mTOR cytoplasmic expression and histological type or grading of carcinomas, degree of tubular formation, anisokaryosis, mitotic activity or lymph node metastasis. In all except one case, the expression pattern of p-mTOR in lymph node metastases was similar or decreased when compared with the primary lesion. The findings suggest that p-mTOR is involved in mammary carcinogenesis in dogs. However, p-mTOR cytoplasmic expression does not appear to be a prognostic indicator in canine mammary carcinomas, which may be related to its subcellular location in the neoplastic cells. Canine mammary tumours may provide a model for the development of innovative medical strategies involving mTOR inhibitors in human breast cancer.



*Keywords:* dog; mammary tumour; phospho-mTOR; PI3K/AKT

## Introduction

The mammalian target of rapamycin (mTOR) is a 290 kDa serine–threonine protein kinase that constitutes an important downstream target of the PI3K/AKT signalling pathway involved in the regulation of overall cellular anabolism, cell growth, proliferation and survival (Carraway and Hidalgo, 2004; Hynes and Boulay, 2006; Monteiro *et al*., 2013).

Aberrant activation of the PI3K/AKT pathway has been documented in many types of human cancer, including breast tumours (Hynes and Boulay, 2006; Miller *et al*., 2011). mTOR is activated by phosphorylation at Ser2448 via the PI3K/AKT signalling pathway and is autophosphorylated at Ser2481 (Nave *et al*., 1999). mTOR activation induces an increase in protein synthesis that is crucial for cell growth and cell cycle progression (Schmelzle and Hall, 2000). Several studies have documented m-TOR activation in breast cancer and associated phospho-mTOR (p-mTOR) expression with worse clinical outcome of the disease, namely shorter disease-free interval and overall survival (Zhou *et al*., 2004; Bose *et al*., 2006; Bakarakos *et al*., 2010).

The PI3K/AKT signalling pathway has generated major interest in recent years, as several of the components of the pathway (including mTOR) have emerged as attractive targets for cancer therapy

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(Carraway and Hidalgo, 2004; Shaw and Cantley, 2006; Miller *et al*., 2011; Mohamed *et al*., 2013). Based on mTOR involvement in the neoplastic transformation process and in tumour growth and progression, targeted therapy against this protein has been under investigation and was shown to decrease tumour growth in some model systems (Mabuchi *et al*., 2009; Chresta *et al*., 2010; Miller *et al*., 2011). Several mTOR inhibitors are currently undergoing evaluation in clinical trials to test their efficacy in the treatment of breast cancer (Alvarez *et al*., 2010; Courtney *et al*., 2010; Miller *et al*., 2011).

In dogs, mTOR activation was recently demonstrated by immunohistochemistry (IHC) in a series of haemangiomas and haemangiosarcomas (Murai *et al*., 2012) and detectable levels of p-mTOR were found in some canine cancer cell lines (Gordon *et al*., 2008; Kent *et al*., 2009) including a mammary carcinoma cell line (Gordon *et al*., 2008; Kent *et al*., 2009; Chen *et al*., 2012). However, to date and to the best of our knowledge, the evaluation of p-mTOR immunoexpression in canine mammary tumours has not been investigated.

The aim of the present study was to evaluate immunohistochemically the expression of p-mTOR in canine mammary carcinomas and to investigate the relationship between this expression and clinicopathological features of the tumours, specifically the histological type and grade of the lesions and the development of lymph node metastases.

## Materials and Methods

### *Specimens*

Forty-five spontaneously arising canine mammary carcinomas and their corresponding regional lymph node metastases ($n = 15$) were included in the study. Samples of normal canine mammary tissue were obtained at necropsy examination from five bitches that were humanely destroyed as part of the national stray dog control programme. All samples were collected from the archives of the Veterinary Pathology Laboratory of Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, and the animal owners gave informed consent to the use of the material for research purposes. All samples were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax. Two consecutive sections (2 μm) were cut from each block, one for haematoxylin and eosin (HE) staining and the other for IHC.

Tumours were classified histologically (Table 1) according to the criteria defined by the World Health Organization classification for mammary tumours of the dog and cat (Misdorp *et al*., 1999). Histological grading of carcinomas was performed according to the Nottingham method proposed by Elston and Ellis (1996) and tumours were classified as grade I (well differentiated), grade II (moderately differentiated) and grade III (poorly differentiated), based on the assessment of three morphological features: tubule formation, anisokaryosis and mitotic activity. A score of 1–3 was given to each feature: tubule formation (1, >75% of tumour area; 2, 10–75% of tumour area; 3, <10% of tumour area); anisokaryosis (1, small, regular and uniform nuclei; 2, increased nuclear size and variation; 3, marked nuclear variation); mitoses per high power (×400) field (1, 0–8 mitoses; 2, 9–17 mitoses; 3, >18 mitoses). The scores for each parameter were added to obtain the overall tumour grade: grade I (well differentiated tumour; 3–5 points), grade II (moderately differentiated tumour; 6–7 points) or grade III (poorly differentiated tumour; 8–9 points).

**Table 1**
**Histological classification of the canine mammary tissues included in the study**

| *Histological classification* | *Number of samples* |
|---|---|
| Normal mammary gland | 5 |
| Malignant tumours | 45 |
| Tubulopapillary carcinoma | 24 |
| Grade I | 11 |
| Grade II | 9 |
| Grade III | 4 |
| Solid carcinoma | 18 |
| Grade I | 3 |
| Grade II | 6 |
| Grade III | 9 |
| Anaplastic carcinoma | 3 |
| Grade I | 0 |
| Grade II | 1 |
| Grade III | 2 |
| Lymph node metastasis | 15 |

### *Immunohistochemistry*

Tissue sections were dewaxed in xylene and hydrated through a decreasing series of alcohol concentrations. Epitope retrieval was heat induced at 99°C in a water bath for 30 min using citrate buffer (10 mM, pH 6.0). After antigen retrieval, endogenous peroxidase activity was blocked with $H_2O_2$ 3% in methanol for 10 min. Tissue sections were then incubated overnight at 4°C with anti-phospho-mTOR ($Ser^{2448}$) rabbit monoclonal antibody (clone 49F9, Cell Signaling Technology, Danvers, Massachusetts, USA), diluted 1 in 150 in a 5% solution of bovine serum albumin (BSA). The primary antibody was detected using a peroxidase-labelled dextran polymer with 3, 3′-