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REVIEW ARTICLE

The importance of the PI3K/AKT/mTOR signaling pathway in canine neoplasms: Literature review

La importancia de la vía PI3K/AKT/mTOR de señalización en las neoplasias caninas: revisión de literatura

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ABSTRACT. The PI3K/AKT/mTOR pathway is related to proliferation, protein synthesis, survival, angiogenesis, apoptosis, and cell motility. Genetic alterations in either activation of oncogenes or inactivation of tumor suppressor make it the second most altered pathway in neoplastic processes. The PI3K/AKT/mTOR pathway is currently considered an attractive target for the development of anti-tumor molecules. Specific inhibitors of this pathway are under development, and those already recognized are being tested in clinical trials, representing a promising approach for the treatment of cancer patients. It is believed that, as this pathway is involved in the development of many human cancers, its activation may also be related to the development of various canine neoplasms. Therefore, this review aims to describe the state-of-the-art knowledge about the PI3K/AKT/mTOR pathway and highlight some research performed with either canine tumors or cellular lines.

Key words: mast cell tumors, osteosarcoma, hemangiosarcoma, mammary carninoma, dog.

RESUMEN. La vía de PI3K/AKT/mTOR se relaciona con la proliferación, la síntesis de proteínas, sobrevida, angiogénesis, apoptosis y la motilidad celular. Alteraciones genéticas, ya sean en la activación de oncogenes o inactivación de genes supresores de tumor hacen de esta la segunda vía más alterada en los procesos neoplásicos. La vía de PI3K/AKT/mTOR actualmente es considerada un objetivo clave para el desarrollo de moléculas antitumorales. Los inhibidores específicos de esta vía se encuentran en desarrollo, y los ya conocidos se encuentran siendo evaluados en ensayos clínicos, lo que representa una alternativa promisoria para el tratamiento de pacientes con cáncer. Se cree que, como esta vía está implicada en el desarrollo de muchos cánceres en humanos, su activación pueda también estar relacionada con el desarrollo de diversos tumores en caninos. Por tanto, esta revisión tiene como objetivo describir el conocimiento actual de la vía PI3K/AKT/mTOR y resaltar algunas investigaciones realizadas, tanto en tumores y linajes celulares caninas.

Palabras clave: mastocitoma, osteosarcoma, hemangiosarcoma, tumor mamario, perros.

INTRODUCTION

Currently, numerous studies have been conducted in both human and veterinary medicine, aiming to unravel the mechanisms that may be involved in the etiology of cancer. This knowledge will lead to the development of new anti-tumor molecules, or even, help select patients with a particular cancer, to undergo therapies already used in patients with other malignancies known to respond to that specific therapy (Mc Auliffe *et al* 2010, Hanahan and Weinberg 2011, Chen *et al* 2012).

In recent years the PI3K/AKT/mTOR (phosphatidyl inositol 3 kinase/ kinase B protein/ rapamycin target in mammals) signaling pathway has attracted the attention of many researchers because it is known that it is implicated in the etiology of various neoplasms. Furthermore, some of the components of this pathway present specific inhibitors that are currently in clinical trial stages (Mc Auliffe *et al* 2010, Ghayad and Cohen 2010).

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Thus, this review describes the PI3K/AKT/mTOR signaling pathway and cites some studies in veterinary medicine performed to assess the expression and efficacy of specific inhibitors in cellular lines or tumors of dogs.

THE PI3K/AKT/MTOR PATHWAY

Hanahan and Weinberg (2011) described ten cellular changes related to malignant transformation of neoplastic cells: genomic instability and mutation, evasion of apoptosis, limitless replicative potential, insensitivity to inhibition of growth factors, continuous angiogenesis, invasion mechanisms and cellular of metastasis, self-sufficiency of growth factors, reorganization of energy metabolism, and evasion of cell destruction by the immune system.

In all these systems, the kinase proteins, when abnormally activated, act on the cell cycle regulation, metabolism, on cell motility, the response to the microenvironment, DNA damage repair and apoptosis. Some studies suggest that these proteins are commonly activated in cancer cells, contributing to cell proliferation and carcinogenesis (Dancey and Sausville 2003, Dillon *et al* 2007, Jiang and Liu 2008).

The PI3K/AKT/mTOR signaling pathway comprises a cascade of serine/threonine kinases that regulate a variety

of cellular processes, including cell cycle progression, cell survival, migration and protein synthesis. Recent evidence has proven that the deregulation of this pathway is associated with promoting tumorigenesis and angiogenesis in various cancers (Jiang and Liu 2009).

Activation of tyrosine-kinase receptors induces the activation of the PI3K/AKT/mTOR pathway (Knowlden *et al* 2008). Once the receptor is activated, the intracellular portion thereof is autophosphorylated and is used as "docking site" for some proteins like PI3K (Marone *et al 2008)*. Once phosphorylated, the PI3K is responsible for the conversion of PIP2 (phosphatidylinositol-4,5-biphosphate) into PIP3 (phosphatidylinositol-3,4,5-triphosphate), thereby recruiting protein that contain homology by pleckistrina (PH), such as AKT and PDK1 (protein-kinase 1 dependent on phospholipase-3) (Martelli *et al* 2007, Kang *et al* 2005). The interaction between the PH domain of AKT and PIP3 promotes conformational changes in the AKT molecule, resulting in the exposure of two phosphorylation sites (Thr 308 in the kinase domain and Ser 473 in the regulatory domain) (Jacinto *et al* 2006).

The PTEN (phosphatase and deleted homologous tensin on chromosome 10) is a tumor suppressor gene, responsible for dephosphorylating PIP3 into PIP2. However, the loss or decreased expression of PTEN indirectly stimulates the activity of PI3K, leading to constitutive activation of AKT and upregulation of mTOR (Mc Auliffe *et al* 2010).

Two main events are responsible for full activation of AKT: in the first, AKT is partially activated by phosphorylation of Thr 308 by PDK1; in the second, full activation of AKT requires phosphorylation of Ser 473 by mTOR-Rictor (Efeyan and Sabatini 2010).

The regulation of mTOR by the AKT protein may occur directly or indirectly (Laplante and Sabatini 2009). In the first case, AKT protein can activate mTOR by phosphorylation of Thr 2446 and Ser 2448 protein domains, while in the second case, it can inhibit the activity of the TSC1/TSC2 complex (hamartin/tuberin)(figure 1) (Zhang *et al* 2003).

The TSC1/TSC2 complex acts as a GTPase activating protein (GAP), inhibiting G Rheb protein (Ras homologue enriched in the brain). AKT disrupts the TSC complex due to the phosphorylation of TSC2, thereby allowing the Rheb to bind to ATP and change from the Rheb-GDP (inactive state) into the Rheb-GTP (active state). GTP-Rheb, in turn, binds to the kinase domain of mTOR-Raptor, and this binding elicits conformational changes in the mTOR-Raptor complex promoting their activation (Zhang *et al* 2003, Inoki *et al* 2002, Long *et al* 2005).

Concomitantly, in situations of low energy intake, LKB1 (serine threonine kinase 11) activates AMPK (protein kinase activator of adenosine monophosphate) protein, which in turn phosphorylates and ends up activating TSC2, thereby inhibiting the activation of mTOR (Corradetti *et al* 2004). Therefore, PI3K/AKT and LKB1/AMPK act as regulators of the mTOR pathway. Both relate to the TSC1/TSC2 complex, but act in opposite ways (figure 1) (Inoki *et al* 2003).

The mTOR protein is a serine/threonine kinase, which is considered a member of the protein kinase family, involved in multiple cellular functions (Zoncu *et al* 2011). This is presented in the form of two complexes: the TORC1 complex, in which mTOR is bound to Raptor (regulatory associated protein TOR) and GβL (β subunit of the G protein) and, the TORC2 complex, in which is mTOR bound to Rictor (protein associated with TOR, insensitive to rapamycin), GβL and mSin 1 (protein kinase associated with activated mitogenic protein 1) (Laplante and Sabatini 2009).

In TORC1 complex, mTOR phosphorylates its effectors S6K1 (ribosomal S6 kinase) and 4EBP1 (eukaryotic initiation factor 4E binding protein). Phosphorylation of 4EBP1 culminates in the release of the previously connected eIF4E (initiation factor of the eukaryotic translation 4E), thereby inducing the activation of eIF4E. Thus, through phosphorylation of its effectors, mTOR influences cell growth and proliferation, by the biosynthesis of proteins, lipids and organelles and also by inhibiting catabolic processes (Laplante and Sabatini 2009). Therefore, the activation of 4EBP1 is often used as a marker of TORC1 activity (figure 1) (Hay and Sonenberg 2004).

The TORC2 complex controls the cytoskeleton actin, in addition to being capable of phosphorylating AKT;

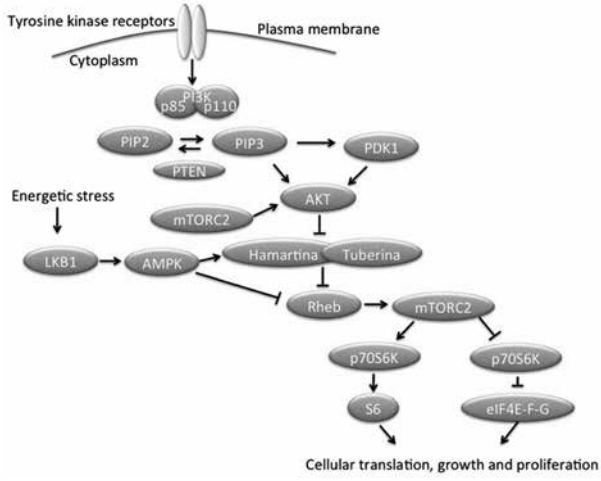

Figure 1. Schematics showing the PI3K/AKT/mTOR pathway. Stimuli originating from PI3K and AMPK can trigger the pathway. Once activated, AKT phosphorylates TSC2, inhibiting the activity of GAP hamartin/tuberin complex and consequently, activate TORC-1. The AMPK, in turn, phosphorylates TSC2 preventing the hamartin/tuberin complex from acting on the GTPase and, therefore, inhibiting mTOR (modified from Miller *et al* 2011).

Esquema que muestra la vía PI3K/AKT/mTOR. Los estímulos procedentes de PI3K y AMPK pueden desencadenar la vía. Una vez activado, fosforila AKT TSC2, inhibiendo la actividad de GAP hamartin/tuberin complejo y, por consiguiente, activar TORC-1. La AMPK, a su vez, fosforila TSC2, evitando el complejo hamartin/tuberin de actuar sobre la GTPasa y, por lo tanto, la inhibición de mTOR (modificado de Miller *et al* 2011).

therefore, some studies suggest that this protein complex corresponds to PDK2, hitherto unknown and responsible for a positive feedback pathway (Foster and Fingar 2010).

The assessment of the PI3K/AKT/mTOR pathway and its aberrant activation have been described in several human cancers, such as breast cancer, colorectal cancer, squamous cell carcinomas, angiosarcomas and soft tissue tumors (Castaneda *et al* 2010, Quesnelle *et al* 2007, Clark *et al* 2010, Lahat *et al* 2010, Dobashi *et al* 2009). After p53, the PI3K/AKT/mTOR is the most frequently activated pathway in neoplastic processes (Agarwal *et al* 2010).

The interest in the components of this pathway has been growing continuously since these are currently considered promising targets for the development of new anticancer molecules. Some of these inhibitors, such as NVP-BKM120, PI3K inhibitor; Perifosine and Triciribine, AKT inhibitors; Temsirolimus, Rapamycin and Everolimus, mTOR inhibitors; and NVP-BEZ235, dual inhibitor of PI3K and mTOR are being used in clinical trials (Ghayad and Cohen 2010).

THE PI3K/AKT/MTOR PATHWAY IN CANINE TUMORS

In veterinary medicine, the activation of this pathway has been extensively studied in canine neoplasms. Some studies were conducted with canine cell lines while others were performed with neoplastic tissues of patients routinely treated in veterinary hospitals. Some of these studies also evaluated the efficacy of specific inhibitors on the studied materials (Chen *et al* 2012, Gordon *et al* 2008, Qiu *et al* 2008[a,b], Kent *et al* 2009, Paolini *et al* 2010, Murai *et al* 2012, Rodriguez *et al* 2012).

OSTEOSARCOMA

The activation of this pathway in canine osteosarcoma cell lines was evaluated by the Western blot technique, in which the presence of both total and phosphorylated mTOR and S6K1 were assessed before and after the cells were exposed to Rapamycin. The cells were also subjected to a clonogenic assay (to assess the ability of colony formation) before and after their exposure to the same drug. This study demonstrated the pathway activation for all studied lines and showed that Rapamycin was able to inhibit mTOR activity. The clonogenic assay demonstrated how effectively this inhibitor reduces survival of tumor cells exposed to the drug, suggesting that this can have a beneficial effect on canine osteosarcoma cells (Gordon *et al* 2008).

A clinical trial was performed using 22 dogs with osteosarcoma that were treated with Rapamycin in doses ranging from 0.01 to 0.08 mg/kg, every 24 hours for 7 days. The animals underwent an incisional biopsy at diagnosis and then, again, within seven days of Rapamycin daily applications. After this period, the animal affected limb was amputated. Tumors from biopsy and amputation were subjected to the electrochemiluminescence technique to detect total and phosphorylated AKT and S6K1. The

Table 1. Use of different types of techiniques for evaluation of PI3K/AKT/mTOR pathway in several neoplasm and its importance as molecular targets.

Uso de diferentes tipos de técnicas para la evaluación de la vía PI3K/AKT/mTOR en diversos neoplamas y su importancia como objetivos moleculares.

Neoplasm	*In vitro/in vivo*	Method	Molecular target
Osteosarcoma	*In vitro*	Western blot (Gordon *et al* 2008)	mTOR (Rapamycin)
	In vivo	Electrochemiluminescence (Paolini *et al* 2010)	mTOR (Rapamycin)
Melanoma	*In vitro*	Western blot (Kent *et al* 2009)	mTOR (Rapamycin)
Hemangiosarcoma	*In vivo (Animal trial)*	Immunohistochemical (Murai *et al* 2012)	AKT (Perifosine or Triciribine) and mTOR (Rapamycin)
Mast cell tumors	*In vivo*	Immunohistochemical (Rodriguez *et al* 2012)	AKT (Perifosine or Triciribine)
Mammary carcinoma	*In vivo*	Immunohistochemical (Qiu *et al* 2008[a,b], Ressel *et al* 2009)	
Various cell lines: B-cell lymphoma, mammary carcinoma, hemangiosarcoma, mast cell tumor, and glioma	*In vitro*	Western blot (Chen *et al* 2012)	AKT (Perifosine or Triciribine) and mTOR (Rapamycin)

phosphorylated/total protein ratio (p-S6K1/S6K1) decreased in the samples treated with the inhibitor, thus showing that Rapamycin is able to modulate the pathway. In this experiment, the drug adverse effects were also evaluated, and it was observed that they were not associated with the used dosage. Therefore, this experiment suggests that Rapamycin may be administered safely and can produce therapeutic concentrations in dogs with osteosarcoma (Paolini *et al* 2010).

MELANOMA

Expression of total and phosphorylated AKT, mTOR, and S6K1 was evaluated in cell lines of canine oral melanoma using the Western blot technique before and after the cells were exposed to Rapamycin. Similarly, the cells were subjected to a clonogenic assay to evaluate the survival fraction before and after exposure to mTOR inhibitor. This study showed that AKT, mTOR and its effector, S6K1, were present and active in cell lines of canine oral melanoma. It also demonstrated that Rapamycin was capable of inhibiting mTOR activity, as well as reducing the survival fraction of the studied cell lines, suggesting that this inhibitor have a beneficial effect on canine oral melanoma cells (Kent *et al* 2009).

HEMANGIOSARCOMA

The immunohistochemical technique was used to assess the expression of the phosphorylated proteins AKT Thr 308, AKT Ser 473, mTOR Ser 2448, 4EBP1 Thr 37/46, and eIF4E in hemangiosarcoma of 37 dogs. The expressions of these same proteins were compared to 27 hemangiomas, four samples of activated endothelial cells present in granulation tissue, and four samples of normal skin tissue. The pathway was activated in 80% of the hemangiosarcomas evaluated and was significantly higher than that found in the hemangiomas and non-neoplastic tissues, suggesting that using specific inhibitors of the pathway would not affect normal dermal tissue and could be beneficial to patients carrying this malignancy (Murai *et al* 2012).

Dickerson and colleagues (2005) studied the presence of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) abnormalities correlated to origin or progression of canine hemangiosarcoma. The mutations found at PTEN C-terminal domain can leverage the cells a survival advantage within their microenvironment. Thus, this was the first study to describe biologically significant mutations of PTEN in the C-terminal domain.

MAST CELL TUMORS

The expression of phosphorylated and total AKT in the Ser 473 domain was evaluated in 25 canine mast cell tumors by immunohistochemical technique. The expression of phosphorylated and total AKT was observed in 25 and 24 samples, respectively. The result indicates that this pathway may be active in canine mast cell tumors (Rodriguez *et al* 2012).

MAMMARY CARCINOMA

The PTEN expression was evaluated in canine mammary carcinomas by the immunohistochemistry technique. The expression was correlated with clinical prognostic factors and survival time. In this study, a positive correlation was demonstrated between the absence of PTEN expression and histological type of tumor, presence of lymphatic invasion, lymph nodes or distant metastasis, tumor differentiation grade, time to recurrence and survival time. Thereby, suggesting that PTEN represents an important prognostic factor in mammary carcinomas of female dogs, corroborates the findings previously demonstrated by Qiu *et al* 2008[a,b] and Ressel *et al* 2009.

VARIOUS CELL LINES

Chen *et al* (2012) used five different canine cell lines from B-cell lymphoma, mammary carcinoma, hemangiosarcoma, mast cell tumor, and glioma, to evaluate the expression of the phosphorylated proteins AKT, mTOR, S6RP, 4EBP1 and eIF4E by Western blot technique. The detectable levels of phosphorylated proteins demonstrated the pathway activation in these cell lines. In this same study, all cell lines were exposed to inhibitors specific to PI3K, AKT and mTOR, which resulted in viability inhibition. The authors also evaluated the efficacy of the combination of inhibitors of PI3K and mTOR in all cell lines. The association between the pathway inhibitors (PI3K, AKT and mTOR) and doxorubicin chemotherapy was evaluated in hemangiosarcoma and breast carcinoma cell lines. The trial results showed that the combination of the two inhibitors resulted in a synergistic effect on the glioma cell line and an additive effect on the other cell lines evaluated. However, the association of the PI3K inhibitor to chemotherapy showed antagonistic effect for the two cell lines evaluated and the association of AKT inhibitor to doxorubicin resulted in synergistic effect in the hemangiosarcoma cell line and antagonistic effect in breast carcinoma. However, the combination of mTOR inhibitor and doxorubicin resulted in an additive effect in both cell lines evaluated. The authors commented on the importance of the pathway in these canine cell lines, highlighting potential therapeutic targets within this pathway (Chen *et al* 2012).

FINAL CONSIDERATIONS

Treatment of cancer patients in veterinary medicine is often a challenge, since many patients still do not have a satisfactory response to therapy. Therefore, research on new therapeutic possibilities with fewer adverse effects

and better results are desirable in veterinary oncology, given the increasing number of cancer patients in small animal clinic.

As well as the human medicine model, where this pathway have been used routinely in several types of cancer due its huge importance in terms of more individual therapeutic target, the evaluation and knowledge of this pathway by veterinarians, becomes a very interesting possibility in the future, since early studies of this pathway have shown promising results to treat neoplastic disorders in small animals.

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