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NEOPLASTIC DISEASE

Dysregulated Expression of Phosphorylated Epidermal Growth Factor Receptor and Phosphatase and Tensin Homologue in Canine Cutaneous Papillomas and Squamous Cell Carcinomas

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Summary

The molecular mechanisms contributing to the development of cutaneous papillomas (CPs) and cutaneous squamous cell carcinomas (CSCCs) are still poorly understood, limiting the ability to identify molecular suitable targets for the development of novel therapies. Persistent activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signalling pathway is a component of epidermal carcinogenesis in dogs. The present study describes the immunohistochemical expression pattern of two key regulatory molecules involved in the PI3K/Akt/mTOR signalling pathway, phosphorylated epidermal growth factor receptor (pEGFR)^{Tyr1068} and phosphatase and tensin homologue (PTEN), in samples of normal canine epidermis, CP, preneoplastic epidermis and CSCC using tissue microarrays to determine whether the deregulated activity of these molecules is involved in the pathogenesis of these relevant epidermal tumours of dogs. Expression of pEGFR and PTEN was dysregulated in most samples of CP, preneoplastic epidermis and CSCC. Overexpression of pEGFR, together with decreased expression of PTEN, may facilitate the progression of some canine CPs and CSCCs by deregulation of the key cellular functions in which the PI3K/Akt/mTOR signalling pathway is involved. These findings suggest that the PI3K/Akt/mTOR signalling molecules may be potential therapeutic targets for canine patients with CP and CSCC.

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Keywords: dog; epidermal carcinogenesis; mTOR signalling pathway; tissue microarray

Introduction

Clinically relevant epidermal tumours in dogs include cutaneous papilloma (CP) and cutaneous squamous cell carcinoma (CSCC) (Gross *et al.*, 2005; Goldschmidt and Goldschmidt, 2016). CP is a common benign tumour of the dog skin usually associated with canine papillomavirus infection (Gross *et al.*, 2005; Goldschmidt and Goldschmidt, 2016). Viral papillomas show typical cytopathic effects associated with a viral aetiology in the keratinocytes, while papillomatous lesions that do not show the typical cellular changes are known as squamous papillomas (Goldschmidt *et al.*, 1988). Despite the fact that most CPs resolve spontaneously within weeks to months, treatment is necessary for some patients (Miller *et al.*, 2013). CSCC is the second most frequent malignant tumour of the canine skin

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(Gross *et al*., 2005; Goldschmidt and Goldschmidt, 2016). Traditional risk factors for CSCC include prolonged sun exposure and unpigmented and lightly haired skin at the tumour site (Goldschmidt and Goldschmidt, 2016). Solar-induced carcinomas are usually preceded by a preneoplastic epidermal lesion known as actinic keratosis, which spontaneously progresses into CSCC (Gross et *al*., 2005). However, CSCCs that are not solar-induced can develop anywhere on the skin, including the subungual region (Gross *et al*., 2005). Although CSCCs are generally locally invasive with metastasis that occurs later in disease, some CSCCs have more aggressive behaviour (Gross *et al*., 2005; Wobeser *et al*., 2007; Miller *et al*., 2013; Goldschmidt and Goldschmidt, 2016). Even though there has been considerable progress in the management of CSCC, treatment options are still limited, and patients with advanced CSCC frequently fail to respond to standard therapies (Hauck, 2012; Miller *et al*., 2013; Goldschmidt and Goldschmidt, 2016).

The molecular mechanisms underlying the development of CP and CSCC in dogs are still poorly understood, limiting our ability to identify molecular targets for the development of novel therapies for these patients. In this regard, we have recently shown that persistent activation of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signalling pathway is a frequent molecular event during canine epidermal carcinogenesis, pointing to this signalling pathway as a potential therapeutic target (Sanz Ressel *et al*., 2019a, b). This signalling pathway has received considerable attention, as the use of mTOR inhibitors have shown promising results in the treatment of CP and CSCC in murine models (Amornphimoltham *et al*., 2004, 2008; Athar and Kopelovich, 2011; Checkley *et al*., 2011, 2014; Callejas-Valera *et al*., 2016). The activation of the PI3K/Akt/mTOR signalling pathway may result from the activation of the epidermal growth factor receptor (EGFR), which leads to its phosphorylation on specific tyrosine residues including tyrosine 1068 (Downward *et al*., 1984). Phosphorylated EGFR (pEGFR) promotes the activation of PI3K, which mediates the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol [3–5]-trisphosphate (PIP3). In turn, PIP3 activates the serine/threonine kinase Akt, which promotes critical cellular processes such as cell cycle progression, cell growth and cell fate decisions by the activation of mTOR. The PI3K/Akt/mTOR signalling pathway is strictly regulated by several negative feedback mechanisms, including the phosphatase and tensin homologue (PTEN), which shuts off PI3K signalling by dephosphorylating PIP3 to PIP2 (Luo *et al*., 2003). The enhanced activity of the PI3K/Akt/mTOR signalling pathway may result from the overactivity of EGFR and/or downregulation of the expression of the tumour suppressor protein PTEN, as it was suggested in man (Molinolo *et al*., 2009; Janus *et al*., 2017). However, the activity of these key regulatory proteins of the PI3K/Akt/mTOR signalling pathway in relevant epidermal tumours of dogs, such as CP and CSCC, is still unknown.

The aim of this study was to evaluate the immunohistochemical expression pattern of two key regulatory molecules involved in the PI3K/Akt/mTOR signalling pathway, pEGFR^{Tyr1068} and PTEN, in samples of normal epidermis, CP, preneoplastic epidermis and CSCC using tissue microarrays (TMAs), in order to determine whether the deregulated expression of these molecules is involved in the pathogenesis of clinically relevant epidermal tumours in dogs.

Materials and Methods

Tissue Microarray

Paraffin wax blocks of formalin-fixed tissues from normal epidermis, CP, preneoplastic epidermis and CSCC of dogs were retrieved from the archives of the Instituto de Patología Dr. Bernardo Epstein, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina. The tissue samples were submitted between 2006 and 2014 and had been processed routinely and embedded in paraffin wax. Sections had been stained with haematoxylin and eosin (HE) for histopathological diagnosis. Clinical information was also available for review.

In order to determine the tissue suitability for inclusion in this study, HE-stained sections from each sample were re-evaluated. A total of 17 samples of normal epidermis, 41 cases of CP, 12 cases of preneoplastic epidermis (actinic keratosis and dysplastic epidermis contiguous to CSCCs) and 150 cases of CSCC were selected. Tumours were classified according to the international histological classification of epithelial tumours of the skin of domestic animals of the World Health Organization (Goldschmidt *et al*., 1988). The distribution of CP cases, based on their histological features, were 28 viral papillomas and 13 squamous papillomas. Each HE-stained slide was mapped under microscopical observation to select and circle with a water-resistant coloured pen the appropriate tissue areas from each donor block to build the TMA.

Three different array blocks were engineered and constructed (the first containing 90 cores from CSCC; the second containing 89 cores from CSCC,

preneoplastic epidermis and normal epidermis; and the last containing 41 cores from CP), taking the approach described by Hewitt (2004), using a semiautomatic tissue arrayer (TMArrayer™, Pathology Devices Inc., San Diego, California, USA), and a core diameter of 1 mm (one core per case). Ten additional samples of normal tissues (i.e. dog skin, human liver, dog lymph node, human thyroid and human cerebellum) and neoplastic tissues (i.e. dog skin, human skin, dog mammary gland, human lung and human colon) were included on each array block as negative and positive controls for immunohistochemistry (IHC) (Hewitt, 2004; Molinolo *et al*., 2007).

HE-stained sections (5 μm) were taken from each TMA to evaluate the tissue preservation and quality of each core. The histological assessment performed showed a good quality of TMA sections. According to the tissue representativity, 15/17 cores of normal epidermis, 36/41 cores of CP, 10/12 cores of preneoplastic epidermis and 140/150 cores of CSCCs were selected for subsequent analyses (Sanz Ressel *et al*., 2019a, b).

Immunohistochemistry

IHC was performed using antibodies against pEGFR^{Tyr1068} (phosphorylated EGFR in Tyr1068, rabbit monoclonal antibody, prediluted; Biocare Medical, Pacheco, California, USA) and PTEN (mouse monoclonal antibody, prediluted; Biocare Medical). The negative control was TMA slides processed for IHC for which the primary antibody was substituted by an isotype-specific immunoglobulin (according to the primary antibody used: rabbit [DA1E] mAb IgG XP® Isotype Control; Cell Signalling Technologies, Danvers, Massachusetts, USA; or mouse [G3A1] mAb IgG1 Isotype Control; Cell Signalling Technologies). A sample of canine CSCC served as a positive control.

TMA sections (5 μm) were placed onto positively charged glass microscope slides. Slides were placed into an oven at 65°C for 30 min to melt the paraffin wax and then immersed in Safeclear II (Fisher Scientific, Hampton, New Hampshire, USA) for 5 min (three times) for dewaxing and hydrated through successive passages in descending concentrations graded alcohols. In order to block endogenous peroxidase, slides were incubated in H_2O_2 3% in 70% ethanol for 30 min and washed three times with distilled water for 5 min. For antigen retrieval, slides were placed in a container with 10 mM sodium citrate and heated in a microwave oven (800 Watts) for 20 min (2 min at 100% power and 18 min at 20% power). The slides were allowed to cool for 15 min at room temperature, washed once with distilled water and then three times with phosphate buffered saline (PBS). Sections were incubated in blocking solution (2.5% bovine serum albumin in PBS) for 30 min at room temperature. Excess blocking solution was discarded and the sections were incubated with the primary antibody diluted in blocking solution at 4°C overnight. After washing with PBS, the slides were sequentially incubated with biotinylated secondary antibody (according to the primary antibody used: biotinylated goat anti-rabbit IgG, BA-1000, diluted 1 in 400; Vector Laboratories, Burlingame, California, USA; or biotinylated goat anti-mouse IgG, diluted 1 in 400; Vector Laboratories) for 30 min. The slides were washed with PBS for 5 min (three times) and incubated with the avidin—biotin complex (Vectastain Elite ABC Kit, PK-6100; Vector Laboratories) for 30 min at room temperature. The slides were washed with PBS and labelling was ‘visualized’ with 3, 3′-diaminobenzidine (SigmaFast™ tablets, D4168-50SET; Sigma Chemical Co., St. Louis, Missouri, USA) to effect. The reaction was stopped in distilled water, and the tissues were counterstained with Mayer’s haematoxylin, dehydrated in graded alcohols and mounted.

Sections were scanned with a ×40 objective using the Aperio CS ScanScope™ (Leica Biosystems Imaging Inc., Buffalo Grove, Illinois, USA). The immunohistochemical expression of pEGFR^{Tyr1068} and PTEN was quantified using the Aperio image analysis algorithms on the ImageScope viewing software. The number of positive epidermal cells was counted for each tissue core, expressing the results as the percentage of positive cells/total cells. According to these results, cores were divided into five categories: 0, <10% of cells labelled; 1, 11—25% of cells labelled; 2, 26—50% of cells labelled; 3, 51—75% of cells labelled; 4, 76—100% of cells labelled (Molinolo *et al*., 2007).

Statistical Analysis

Differences in immunohistochemical reactivity between groups were analysed using the Kruskall—Wallis test for non-Gaussian populations (non-parametric analysis of variance; ANOVA) followed by the Dunn’s post-test (Statgraphics™ software, The Plains, Virginia, USA; $P < 0.05$ and 95% confidence).

Results

Expression of pEGFR^{Tyr1068} and PTEN in Normal Epidermis

To determine whether expression of pEGFR^{Tyr1068} and PTEN is dysregulated in canine epidermal tu-

mours, we started by evaluating the expression of these proteins in normal epidermis (Fig. 1; Supplementary Table 1). The immunodetection of pEGFR^{Tyr1068} showed a high number of positive epidermal cells with membrane and cytoplasmic labelling limited to the basal and spinous cell strata (Fig. 2). PTEN immunolabelling revealed a low number of positive epidermal cells located in the basal and spinous cell strata, showing a cytoplasmic expression pattern (Fig. 3).

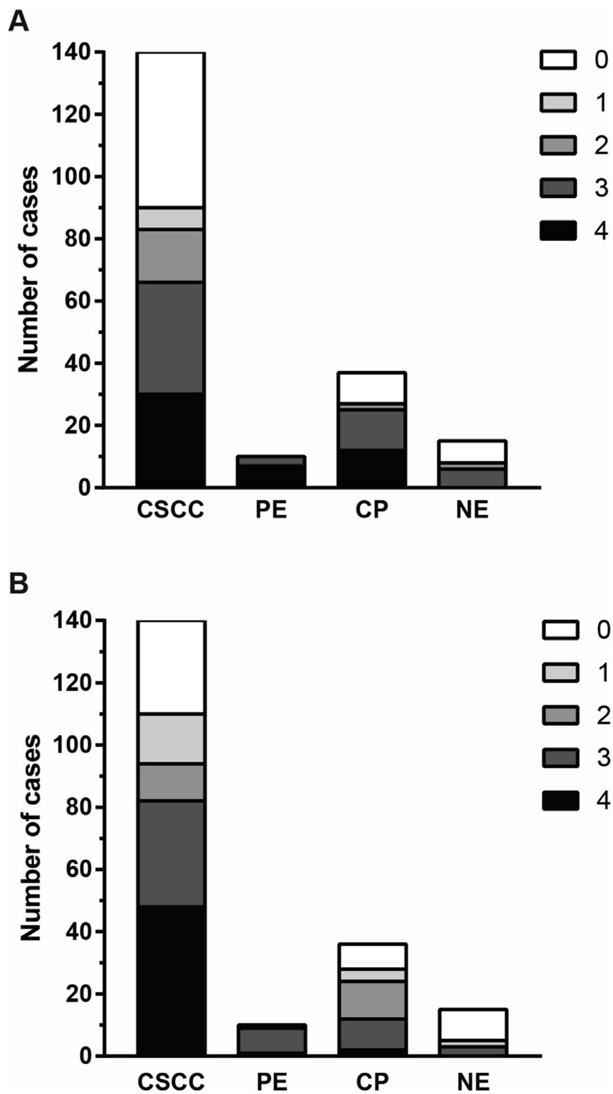

Fig. 1. Quantitative analysis of immunohistochemistry for pEGFR^{Tyr1068} (A) and PTEN (B) in samples of normal epidermis (NE), cutaneous papillomas (CP), preneoplastic epidermis (PE) and cutaneous squamous cell carcinomas (CSCC). The number of positive epidermal cells was expressed as the percentage of positive cells/total cells. Score: 0, <10% of cells labelled; 1, 11–25% of cells labelled; 2, 26–50% of cells labelled; 3, 51–75% of cells labelled; 4, between 76 and 100% of cells labelled.

Expression of pEGFR^{Tyr1068} and PTEN in Cutaneous Papilloma

CP samples showed a high number of epidermal cells positive for pEGFR^{Tyr1068} and PTEN (Fig. 1; Supplementary Table 2). pEGFR^{Tyr1068} labelling revealed a membrane and cytoplasmic expression pattern limited to the basal and spinous strata (Fig. 2). PTEN immunolabelling showed a high cytoplasmic expression limited to the basal and spinous strata in most samples (Fig. 3), although low expression of this protein was observed in some samples (12/36, 33.33%). The Kruskall–Wallis test showed no significant differences in the percentage of labelled cells between normal epidermis and CP for pEGFR^{Tyr1068} or PTEN. Furthermore, no significant differences for the selected regulatory proteins of the PI3K/Akt/mTOR signalling pathway were found between viral papillomas and squamous papillomas.

Expression of pEGFR^{Tyr1068} and PTEN in Preneoplastic Epidermis and Cutaneous Squamous Cell Carcinoma

We finally explored the expression of pEGFR^{Tyr1068} and PTEN in samples of preneoplastic epidermis and CSCC (Fig. 1; Supplementary Tables 3 and 4). pEGFR^{Tyr1068} immunoreactivity was expressed in a high number of epidermal cells in all preneoplastic epidermal samples and in 90/140 (64.28%) CSCC samples, showing membrane and cytoplasmic immunoreactivity in the epidermal cells of the basal and spinous strata (Fig. 2). The immunodetection of PTEN showed a high number of positive epidermal cells in all preneoplastic epidermis samples and in 111/140 (79.29%) CSCC samples, for which cytoplasmic expression in the basal and spinous cell strata was observed (Fig. 3). The Kruskall–Wallis test showed significant differences in the percentage of cells labelled for pEGFR^{Tyr1068} between normal epidermis and preneoplastic epidermis (P <0.01), while no significant difference was found between normal epidermis and CSCC. Furthermore, significant differences in the percentage of cells expressing PTEN were found between normal epidermis and preneoplastic epidermis (P <0.01) and between normal epidermis and CSCC (P <0.01). PTEN showed no significant differences between CSCCs based on tumour location.

Discussion

The molecular mechanisms involved in the development of CP and CSCC in dogs are still poorly understood, limiting the ability to identify molecular targets for the development of novel therapies for these patients. In this regard, characterization of relevant

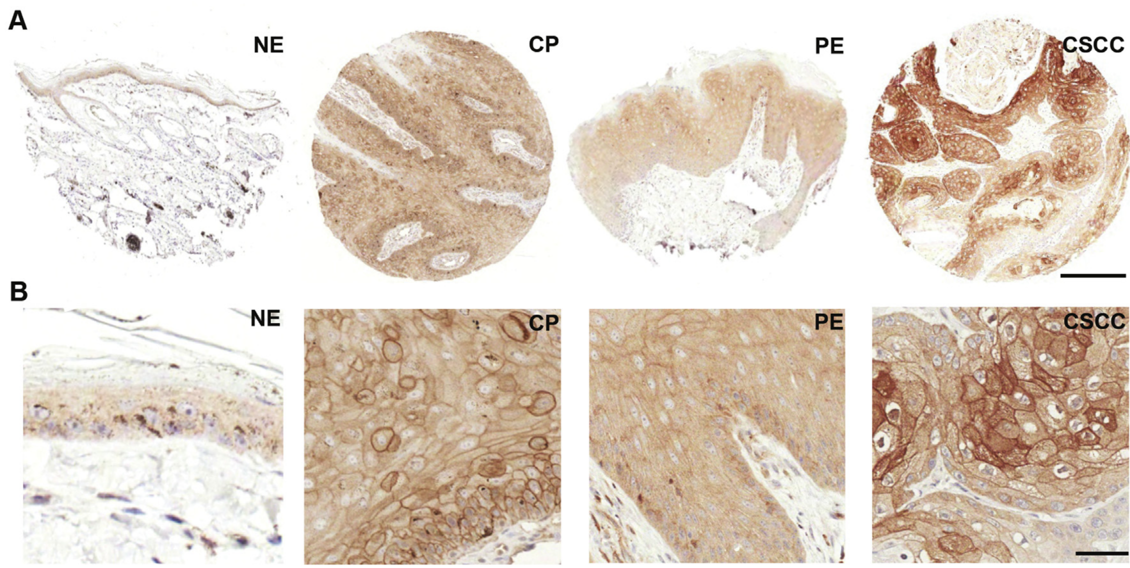

Fig. 2. (A) Immunodetection of pEGFR^{Tyr1068} in array cores of normal epidermis (NE), cutaneous papillomas (CP), preneoplastic epidermis (PE) and cutaneous squamous cell carcinomas (CSCC). (B) pEGFR^{Tyr1068} -positive cells (brown) show strong membrane labelling and a cytoplasmic labelling pattern limited to the basal and spinous cell strata in samples of normal epidermis, CP, preneoplastic epidermis and CSCC. Bars, 300 μm (A) and 50 μm (B).

signalling molecules in a large number of tumour samples under identical and standardized conditions can help to identify the key molecular events involved in canine epidermal tumourigenesis. In this study, we have described the immunohistochemical expression pattern of two key regulatory proteins of the PI3K/Akt/mTOR signalling pathway, pEGFR^{Tyr1068} and PTEN, in normal canine epidermis, CP, preneoplastic epidermis and CSCC using TMA in order to determine whether the dysregulated expression of these molecules is involved in epidermal tumourigenesis in dogs.

The immunodetection of the selected regulatory proteins of the PI3K/Akt/mTOR signalling pathway in CP showed that most samples displayed a high level of expression of pEGFR^{Tyr1068}, although low

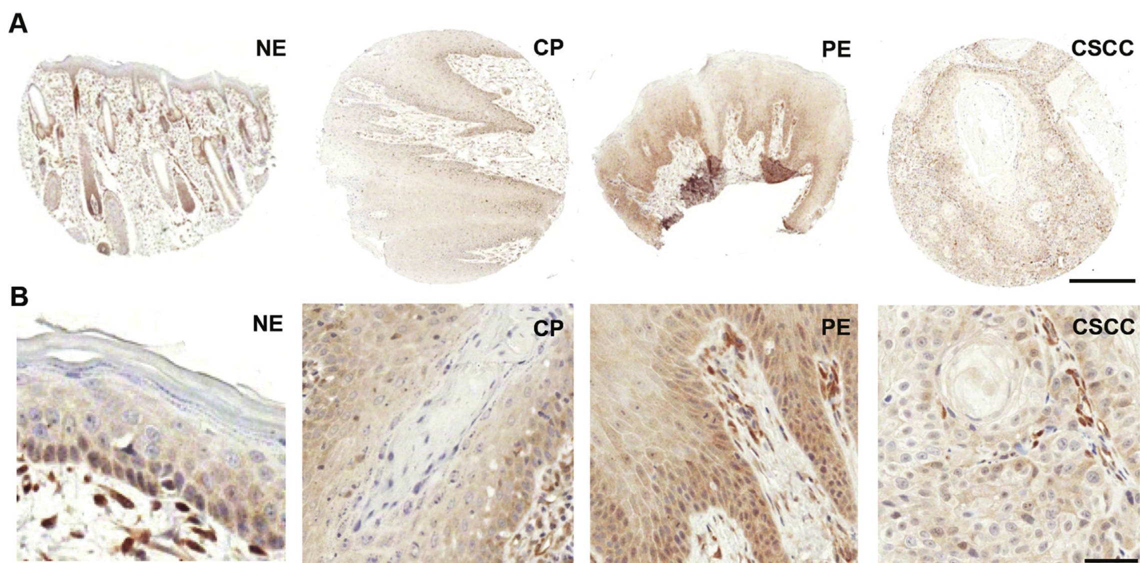

Fig. 3. (A) Immunodetection of PTEN in array cores of preneoplastic epidermis (NE), cutaneous papillomas (CP), preneoplastic epidermis (PE) and cutaneous squamous cell carcinomas (CSCC). (B) PTEN immunolabelling with a cytoplasmic reaction (brown) in the basal and spinous cell strata in samples of normal epidermis, CP, preneoplastic epidermis and CSCC. Bars, 300 μm (A) and 50 μm (B).

expression of this protein was also observed in some samples. In this regard, previous studies have shown that EGFR plays an important role in epidermal tumourigenesis mediated by human papillomavirus (HPV). Indeed, keratinocytes immortalized by HPV exhibit the constitutive activation of EGFR *in vitro* (Zyzak *et al.*, 1994), while pharmacological inhibition of this receptor effectively blocks the growth and survival of immortal keratinocytes in culture (Ben-Bassat *et al.*, 1997, 1999). Similarly, Woodworth *et al.* (2000) showed that EGFR activation is important for the immortalization of mouse keratinocytes by HPV, as well as for the progression of these immortal cells to papillomas and carcinomas. However, the fact that some papillomas showed reduced or absent expression of $pEGFR^{Tyr1068}$ indicates that, although EGFR activation could play an important role in the development of canine CP, it may not be strictly necessary. Indeed, all of the cases in which the pEGFR was not detected were tumour samples in which the mTOR signalling pathway was persistently activated (Sanz Ressel *et al.*, 2019b). These findings are aligned with those observed in mouse keratinocytes immortalized by HPV, where treatment with the tumour promoter 12-O-tetradecanoylphorbol-13-acetate induced the formation of papillomas even in grafts containing keratinocytes not expressing EGFR (Woodworth *et al.*, 2000). These results suggest that the tumour progression of keratinocytes can also occur through pathways independent of EGFR activation (Woodworth *et al.*, 2000). Similarly, the results obtained from study of a murine model of epidermal carcinogenesis suggest that once a determined stage of tumour growth has been reached, the survival of the tumour cells can be achieved by the activity of parallel growth promoter pathways independent of EGFR (Dahlhoff *et al.*, 2011). Altogether these results provide further evidence of the existence of still poorly understood molecular alterations in the pathogenesis of CP in dogs, which may lead to the EGFR-independent activation of growth promoter signalling pathways such as the mTOR signalling pathway.

PTEN was expressed in the cytoplasm of cells of the basal and spinous strata in most CP samples, but low expression of this protein was observed in some samples. These findings may be related to the attempt carried out by the tissue to restore the normal functioning of the PI3K/Akt/mTOR signalling pathway during epidermal tumourigenesis, while the tumour suppressor functions of this molecule may be compromised in some patients. Similarly, previous studies in mice have shown that the decreased activity of PTEN may be a frequent event during the tumour progression of epidermal keratinocytes (Segrelles *et al.*, 2002). In this regard, Suzuki *et al.* (2003) demonstrated through a genetically modified mouse model that the deficiency of PTEN leads to a hyperproliferative epidermis and, consequently, to the development of spontaneous tumours such as papillomas. In this model, increased proliferation of the epidermal keratinocyte was related to an increased activity of the PI3K/Akt/mTOR signalling pathway (Suzuki *et al.*, 2003). In connection with the findings that show that a reduced expression of PTEN may play an important role during epidermal tumourigenesis by the persistent activation of the PI3K/Akt/mTOR signalling pathway, we observed that the tumours exhibiting reduced PTEN labelling were positive for the phosphoproteins of the mTOR signalling pathway (Sanz Ressel *et al.*, 2019b).

We then explored the expression pattern of the two regulatory proteins of the PI3K/Akt/mTOR signalling pathway in samples of preneoplastic epidermis and CSCC. The immunodetection of $pEGFR^{Tyr1068}$ showed that the persistent activation of this receptor is a frequent event in most canine samples of preneoplastic epidermis and CSCC, similar to previous results reported in man (Kikuchi *et al.*, 1990; Nazmi *et al.*, 1990). However, Uribe and Gonzalez (2011) showed that only 73% of human CSCCs analysed overexpressed EGFR. Similarly, Fogarty *et al.* (2007) showed that only 43% of human CSCCs examined showed EGFR expression and only 53% of those showed activation of the receptor. These observations are aligned with a recent report describing that the EGFR was expressed in 90% of human CSCCs evaluated, but only 35% of them showed overexpression (Cañueto *et al.*, 2017). These findings are similar to those of the present study, in which only 64% of CSCCs showed expression of $pEGFR^{Tyr1068}$. These results suggest that the activation of EGFR is an important event during the malignant transformation of epidermal keratinocytes in dogs, but it may not be strictly necessary. Furthermore, a high level of EGFR activation may not be required for PI3K/Akt/mTOR activation (Joseph *et al.*, 2014). In this regard, we recently showed that the activation of pS6, the downstream target of the PI3K/Akt/mTOR signalling pathway, increased in most CSCCs (Sanz Ressel *et al.*, 2019a). We now show that the enhanced activity of the mTOR signalling pathway may not be correlated with the activity of EGFR, since some of the cases in which pS6 was detected were tumour samples in which EGFR was not persistently activated. These findings are aligned with those reported in human squamous cell carcinomas of the head and neck (Molinolo *et al.*, 2007), which raises the question of the possible existence of EGFR-independent routes that can promote the growth and survival of tumour

cells. Altogether, these findings suggest that EGFR could represent a potential therapeutic target only in CSCC canine patients with activation of this receptor.

The immunodetection of PTEN showed high expression in all preneoplastic epidermal samples, while its expression was reduced or absent in some CSCC samples. These findings may be related to an attempt by the tissue to restore the normal functioning of the PI3K/Akt/mTOR signalling pathway during the early stages of malignant transformation of canine epidermal keratinocytes. These results disagree with those reported in mice, which suggest that PTEN is not important during the initial stages of murine epidermal carcinogenesis (Segrelles *et al*., 2002). Furthermore, the fact that PTEN expression was reduced or absent in some CSCC samples indicates that the tumour suppressor functions of this molecule may be compromised during the malignant transformation of epidermal keratinocytes, similar to that previously reported in mice (Segrelles *et al*., 2002). Likewise, the absence of PTEN was related to the development of spontaneously arising cutaneous squamous cell carcinomas in a murine model of skin carcinogenesis (Suzuki *et al*., 2003). In this model, increased proliferation and resistance to apoptosis of epidermal keratinocytes was associated with increased activity of downstream molecules of the mTOR signalling pathway (Suzuki *et al*., 2003). The fact that we have previously shown that most of these CSCC patients overexpressed pS6 (Sanz Ressel *et al*., 2019a) supports the hypothesis that the decrease of PTEN expression at the tumour stage may cause the overactivity of the PI3K/Akt/mTOR signalling pathway in some animals and, consequently, the deregulation of cellular processes in which this signalling pathway is involved.

In summary, we show here that the expression of pEGFR and PTEN is dysregulated during epidermal carcinogenesis in dogs. In this regard, overexpression of pEGFR and/or the decrease of expression of PTEN may facilitate the progression of canine CP and CSCC by deregulation of the key cellular functions in which the PI3K/Akt/mTOR signalling pathway is involved. Furthermore, the fact that EGFR activation was absent in some canine patients suggests that therapeutic approaches aimed at modulating the growth and survival of tumour cells by the use of EGFR inhibitors would be ineffective as sole therapeutic agents in some CP and CSCC patients, as it was previously suggested in man (Molinolo *et al*., 2007).

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Conflict of Interest Statement

None of the authors of this paper have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcpa.2019.10.005.

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