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NEOPLASTIC DISEASE

Overexpression of Ephrin A3 Receptor in Canine Prostatic Carcinoma

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Summary

Ephrin A3 (EphA3), a member of the ephrin receptor tyrosine kinase family, is involved in a variety of functions in normal cells, especially during embryonic development, and alterations in its expression profile have been observed in several human cancers. However, there are no reports of the expression of EphA3 in normal, hyperplastic or neoplastic canine prostate tissue or in other types of canine tumours. Six normal, 15 hyperplastic and 21 neoplastic canine prostates were examined immunohistochemically with a polyclonal antibody specific for human EphA3. The percentage of positive cells in all prostatic carcinomas was increased, with a mean of 89.28 ± 5.18% compared with normal (9.17 ± 6.72%) and hyperplastic prostates (20.00 ± 8.28%). EphA3 expression was not correlated with the histological subtypes of prostate cancer or with the Gleason score. The increase in EphA3 expression in canine prostatic carcinomas suggests the involvement of this receptor in prostatic carcinogenesis and its potential use as a target for new therapeutic strategies.

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Introduction

Prostatic carcinoma (PC) in dogs is a highly aggressive cancer, characterized by a high rate of metastasis at presentation (Cornell *et al.*, 2000). The prognosis is considered poor and most affected dogs are not treated due to the poor quality of life. Radical prostatectomy is usually associated with a very high incidence of postoperative urinary incontinence (Goldsmid and Bellenger, 1991). Radiotherapy has been attempted in combination with surgery, but with disappointing results and severe adverse effects (Turrel, 1987). No effective chemotherapeutic protocols are available for the treatment of PC in dogs. Therefore, research to develop better treatment options and, possibly, translation of new and successful therapeutic strategies already available in human oncology is needed.

With 16 members, the ephrin receptor family is the largest family of tyrosine kinase receptors and it is of increasing interest in developmental therapeutics. ephrin receptors are divided into two groups, A and B, based on sequence homology and binding affinities to A or B class ephrin ligands (Pasquale, 2005). They are expressed widely during embryogenesis and regulate developmental processes such as axon guidance, angiogenesis and boundary formation (Pasquale, 2008). Many of the effects are mediated by signalling cascades modulating cell adhesion or cell movement, although they may also control cell survival, proliferation and differentiation (Himanen *et al.*, 2007). The most promising molecule as tumour marker and potential therapeutic target in cancer, ephrin A3 (EphA3), is highly expressed at various stages of embryonic development of the brain and spinal cord, lungs, kidney, heart and musculature (Kilpatrick *et al.*, 1996). After the embryonic period, its expression declines, being usually low, if detectable at all, in

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adults. However, EphA3 becomes re-expressed in a wide range of epithelial and mesenchymal tumours, and often correlates with a more aggressive behaviour and poor prognosis (Janes *et al*., 2014). During carcinogenesis, EphA3 plays an important role in a variety of biological functions, such as tumour cell proliferation, angiogenesis and tumour progression (Keane *et al*., 2012). The role of EphA3 in canine oncology has not been investigated, and even in men, the number of studies focussing on this molecule in prostate cancer is limited (Fox *et al*., 2006; Singh *et al*., 2008; Wu *et al*., 2014).

Therefore, the aim of the present study was to evaluate the immunohistochemical expression and distribution of EphA3 in normal, hyperplastic and neoplastic canine prostates and the potential association with the biological behaviour and the acquisition of an aggressive phenotype.

Materials and Methods

Samples and Histopathology

Forty-two, formalin-fixed and paraffin wax-embedded canine prostatic samples were selected from the archive of the diagnostic pathology service of the School of Veterinary Science, The University of Queensland, Australia ($n = 36$), and the Universidade Estadual Paulista, Brazil ($n = 6$). Sections (5 μm) were stained with haematoxylin and eosin (HE) for histopathological examination. The samples were classified as normal prostates, benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC). The diagnosis of PC was limited to those cases showing no evidence of urinary bladder involvement. PCs were classified according to the growth patterns described by the human WHO classification of tumours of the urinary system and male genital organs (Eble *et al*., 2004) and recently adapted to canine PCs (Palmieri *et al*., 2014). Gleason grading was performed according to the 2005 International Society for Urological Pathology modified grading system (Epstein *et al*., 2005), recently described in dogs (Palmieri and Grieco, 2015). Gleason grading assigns a numerical grade (1–5) based on the architectural patterns of the tumour, with patterns 4 and 5 showing increasingly abnormal glandular architecture (Gleason, 1966). The sum of the primary grade assigned to the most prevalent pattern and the secondary grade assigned to the second most prevalent pattern provides the overall Gleason score (GS). If there is only one pattern, its grade is doubled to reach the score (Gleason, 1966). The GS is related to the biological behaviour of human PCs, so that men with tumours of GS 9 to 10 have a significantly worse prognosis than men with tumours of GS 8 or less in terms of biochemical recurrence (Pierorazio *et al*., 2013).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using an indirect avidin–biotin–peroxidase procedure. All the incubations were performed at room temperature unless otherwise stated. Following dewaxing and rehydration of the sections, endogenous peroxidase was neutralized by incubation in H_2O_2 3% in methanol for 30 min. Antigen retrieval was achieved by submerging the sections in 0.1 M sodium citrate (pH 6.0) and subsequent heating in a microwave oven for 15 min and cooling for 20 min. The sections were pre-incubated with 5% normal goat serum and avidin–biotin blocking solution. Sections were then incubated with the primary rabbit polyclonal anti-EphA3 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA; dilution 1 in 800) overnight and with a biotinylated secondary antibody (Vector Laboratories, Burlingame, California, USA) in phosphate buffered saline (PBS). Following incubation with peroxidase-conjugated avidin–biotin complex (Vector Laboratories) for 30 min, as indicated by the manufacturer, peroxidase activity was 'visualized' by use of 3′, 3′-diaminobenzidine for 5 min in the dark. Slides were then counterstained with Mayer's haematoxylin, dehydrated and mounted. As a negative control, primary antibodies were substituted with PBS, while normal canine skin was used as a positive control.

Evaluation of Immunohistochemistry

A semiquantitative immunohistochemical assessment was performed analysing 10 high-power fields (×400). Samples were subdivided based on the protein expression levels into five ranges (semiquantitative score): 0, no positive cells; 1+, >0 to ≤10% positive cells; 2+, >10% to ≤25% positive cells; 3+, >25% to ≤50% positive cells; 4+, >50% to ≤75% positive cells; and 5+, >75% positive cells. The labelling intensity (qualitative score) was recorded as negative (0) or positive on a scale from weak (+) to strong (+++).

Statistical Analysis

Differences between normal prostates, BPH, PC, each different subtype of PC and each different GS were assessed by the Chi-square test and considered to be significant at $P < 0.005$.

Results

Histopathology

The histopathological examination confirmed that samples comprised of six normal prostates, 15 cases

of BPH and 21 cases of PC. Four growth patterns of PC were differentiated: (1) cribriform ($n = 12$) (Fig. 1A) where ducts were expanded by neoplastic cells forming irregular fenestrae, often associated with central necrosis; (2) solid ($n = 5$) (Fig. 1B) formed of solid sheets, cord of cells or isolated individual cells without any specific growth patterns; (3) papillary ($n = 2$) (Fig. 1C) formed of dilated ducts containing papillary projections of neoplastic cells; and (4) small acinar/ductal ($n = 2$) (Fig. 1D) where neoplastic cells formed small acini and tubules.

Fifteen dogs were classified as having tumours with a GS of 10 (10 cribriform with necrosis; five solid), four as having tumours with a GS of 9 (two cribiform without necrosis in a mixed tumour, one papillary in a mixed tumour and one small acinar/ductal in a mixed tumour) and two as having tumours with a GS of 8 (one small acinar/ductal and one papillary).

Immunohistochemistry

Moderate cytoplasmic expression of EphA3 was demonstrated in few randomly scattered epithelial cells in five of six normal prostates (Fig. 2A). The percentage of positive cells ranged from 5 to 20%, with a mean of 9.17 ± 6.72%. Other EphA3-positive cells included endothelial cells, fibroblasts and smooth muscle cells within the stroma.

All cases of BPH were positive with weak (5/15) or moderate (10/15) labelling intensity (Fig. 2B). The percentage of positive cells ranged from 5 to 35%, with a mean of 20 ± 8.28%.

In all PC samples, the antibody showed diffuse and moderate (4/21) to strong (17/21) labelling (Figs. 2A and 3B). In two cases, membranous expression was also evident (Fig. 3C). The mean percentage of neoplastic epithelial cells labelled was 89.28 ± 5.18%. When present, cancer glands circumferentially encircling a nerve fibre (perineurial invasion) were strongly positive (Fig. 3D).

A significant difference in the semiquantitative score was observed between normal/hyperplastic prostates and PCs (P <0.005). Labelling intensity and percentage of positive cells were similar in all histological subtypes without any significant differences.

The mean percentage of positive cells in tumours of GS 10 (92.55 ± 7.82%) and GS 9 (91.47 ± 5.67%) were slightly higher than in tumours of GS 8 (89 ± 6.11%), although the difference was not significant.

Discussion

The present study represents the first investigation of EphA3 expression in a canine solid tumour, and specifically in canine prostate cancer. The increased immunohistochemical labelling for EphA3 in canine PC cells compared with normal and hyperplastic

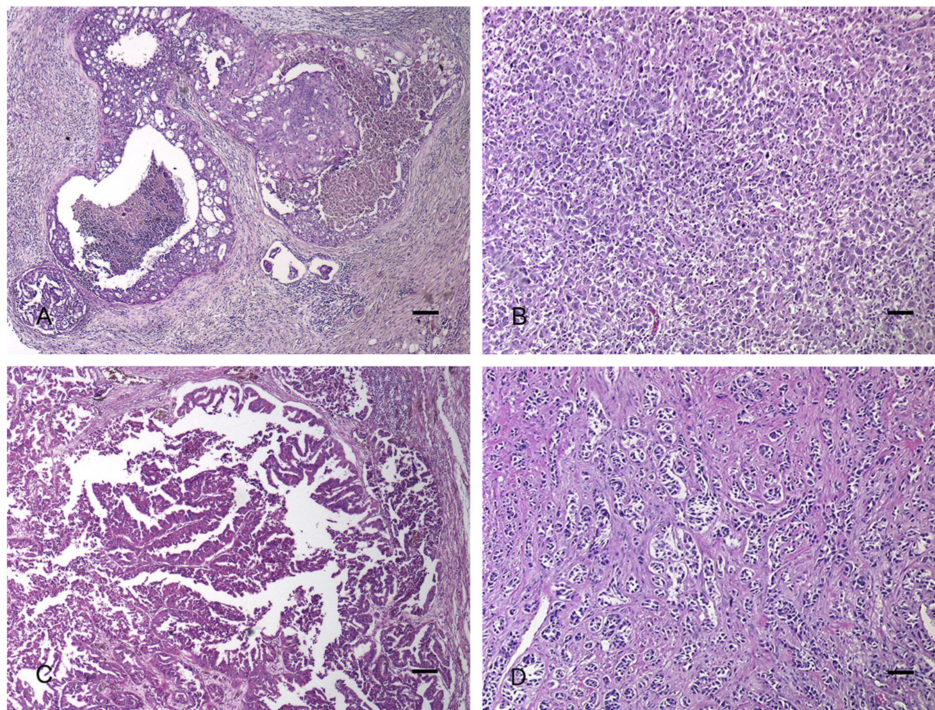

Fig. 1. Histological growth pattern of canine prostatic carcinoma. (A) Cribriform with central necrosis. HE. Bar, 200 μm. (B) Solid undifferentiated. HE. Bar, 50 μm. (C) Papillary. HE. Bar, 200 μm. (D) Small acinar/ductal. HE. Bar, 50 μm.

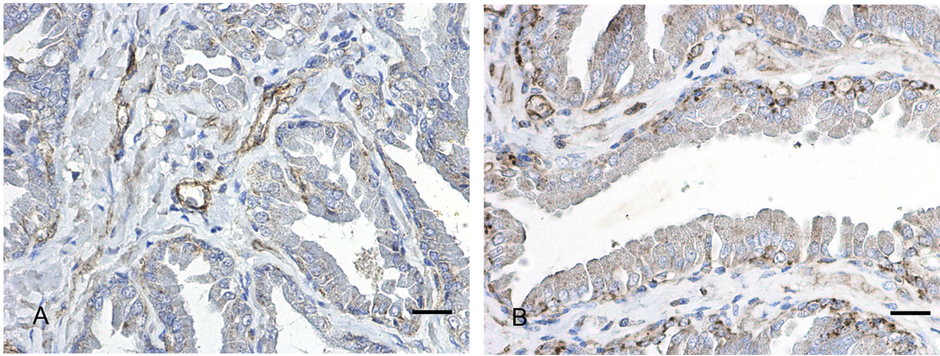

Fig. 2. (A) Normal prostate showing randomly scattered basal cells with strong cytoplasmic EphA3 expression. Note the positive reaction of endothelial cells and fibroblasts within the stroma. IHC. Bar, 80 μm. (B) Benign prostatic hyperplasia showing strong EphA3 expression in the basal cell layer. IHC. Bar, 80 μm.

canine prostates suggests that EphA3 may play a role in the carcinogenesis of prostate cancer in dogs, as already demonstrated in human hepatic cancer, lung cancer, renal cancer, colorectal cancer, melanoma, sarcoma, rhabdomyosarcoma, glioblastoma and gastric cancer (Chiari *et al*., 2000; Hafner *et al*., 2004; Wimmer-Kleikamp and Lackmann, 2005; Clifford *et al*., 2008; Bae *et al*., 2009; Valsesia *et al*., 2011; Xi *et al*., 2012; Day *et al*., 2013).

Similar findings have been obtained by Wu *et al*. (2014) in human prostate cancer, with EphA3 expression detected in PC tissues and not in adjacent normal tissues or BPH tissue specimens, as well as in PC cell lines with different metastatic potential, namely LNCaP (androgen-dependent, non-metastatic and weakly tumourigenic) and C4–2B (androgen-independent, metastatic) cells.

In normal and hyperplastic prostates, EphA3 is mainly expressed by few cells of the basal compartment that possesses self-renewal capacity (Goldstein *et al*., 2010). Basal cells are less differentiated and proliferate more frequently compared with secretory cells (Bonkhoff *et al*., 1994), and their role in prostatic carcinogenesis has been discussed in dogs, with 25% of canine PCs expressing the basal cell marker cytokeratin 5 (Akter *et al*., 2015). In human solid tumours,

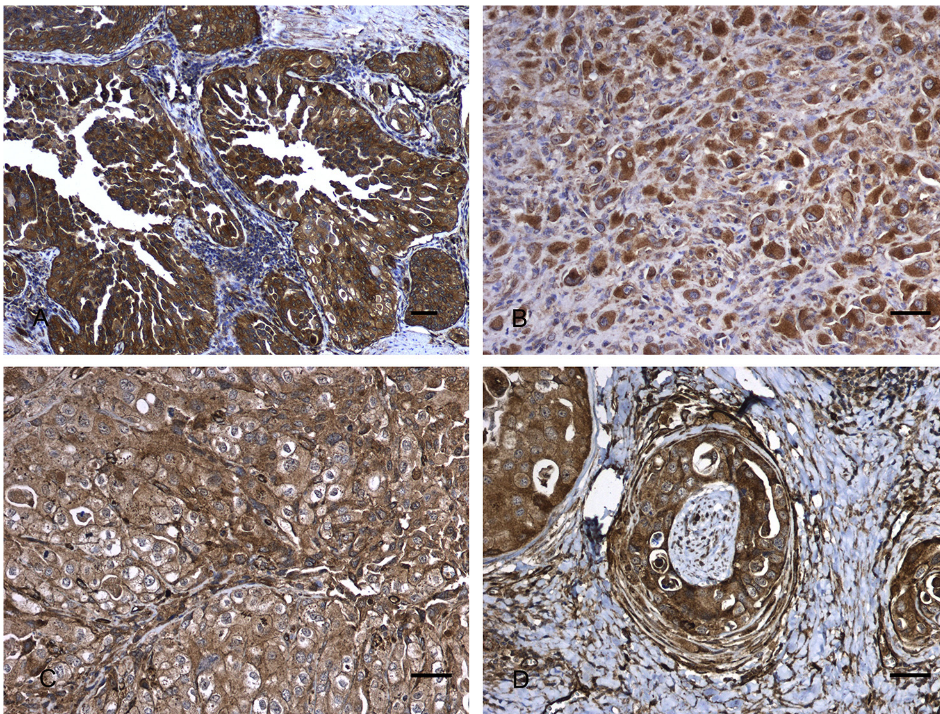

Fig. 3. Immunohistochemical expression of EphA3 in canine prostatic carcinomas (PCs). (A) Cribriform PC. IHC. Bar, 50 μm. (B) Solid PC. IHC. Bar, 100 μm. (C) Cribriform PC showing cytoplasmic and membranous labelling. IHC. Bar, 100 μm. (D) Neoplastic cells encircling a nerve fibre (perineurial invasion) showing diffuse cytoplasmic expression of EphA3. IHC. Bar, 100 μm.

EphA3 is expressed by both cancer stem cells and mesenchymal stem cells within the tumour stroma, thereby indicating a role for EphA3 in both tumour initiation and long-term maintenance (Janes *et al.*, 2014). This supports the preferential expression of EphA3 in the less differentiated and stem cell-like canine prostatic basal cell layer.

The present results reveal that EphA3 expression is not correlated with the histological subtype of canine prostate cancer or with the GS. However, canine PC is a highly aggressive tumour, mostly of GS 10 (Palmieri and Grieco, 2015) and in the present study only three categories of GS (8, 9 and 10) were considered. No information is available on the expression pattern of EphA3 in low GS prostatic cancers, although a slightly increased EphA3 was observed in tumours of GS 10 and GS 9 compared with those of GS 8. A positive correlation between the levels of EphA3 and the GS of PCs has been identified in human clinical specimens (Wu *et al.*, 2014). This is consistent with data from melanoma and breast cancer, where EphA3 is reported to be highly expressed in metastatic lesions, but not in the primary tumours (Easty and Bennett, 2000; Vecchi *et al.*, 2008). In colorectal cancer, the expression of EphA3 positively correlated with tumour size, histological grade, depth of invasion, lymph node metastasis, distant metastasis and TNM stage (Xi and Zhao, 2011). Regarding PCs, *EphA3* has been identified as one of several genes associated with androgen independence, with expression of the gene being increased 39-fold in androgen-independent PC cells (LNCaP-C33) compared with in androgen-dependent PC cells (LNCaP-C33) (Singh *et al.*, 2008). Human PC is initially androgen sensitive and relapses after androgen deprivation therapy as a hormone-refractory, highly undifferentiated, highly aggressive and heterogeneous tumour (Shah *et al.*, 2004). In dogs, PC is not responsive to androgen deprivation therapy and has an undifferentiated morphology and aggressive behaviour, resembling the refractory phase of human PC (Lai *et al.*, 2008). Therefore, the increased expression of EphA3 in the present prostatic samples is consistent with the androgen-independent feature of both canine and human PC.

Accumulated research has shown that the aberrant regulation of *EphA3* and its genetic alteration are involved in the development and progression of various cancers (Bae *et al.*, 2009). In many cancers, especially haematopoietic tumours, abnormal *EphA3* expression is predominantly overexpression of non-mutated EphA3 protein (Keane *et al.*, 2012). The high level of expression of EphA3 in canine prostate cancer might be due to a mechanism of gene amplification and overexpression or the effect of its sequence alteration on transcriptional control, such as DNA methylation.

Based on these preliminary findings, we suggest that *EphA3* may be a potential oncogene that has an important role in the development and malignant progression of canine PC and, therefore, may be a useful therapeutic target. The potential of EphA3 as a target for cancer therapy has been demonstrated previously (Brantley *et al.*, 2002; Vearing *et al.*, 2005; Vail *et al.*, 2014). Soluble EphA3-Fc receptors and anti-EphA3 antibody can inhibit proliferation of tumour cells and decrease tumour volume *in vivo* (Brantley *et al.*, 2002; Vearing *et al.*, 2005). Moreover, targeting EphA3 inhibits cancer growth by disrupting the integrity and function of newly formed tumour stroma and microvasculature (Vail *et al.*, 2014).

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