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Alterations in PTEN, MDM2, TP53 and AR protein and gene expression are associated with canine prostate carcinogenesis

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ABSTRACT

The PTEN, AR, MDM2 and p53 protein network plays a central role in the development of many human cancers, thus eliciting the development of targeted cancer therapeutics. Dogs spontaneously develop tumours, and they are considered a good model for comparative oncology initiatives. Due to the limited information on these proteins in canine tumours, this study aimed to investigate gene and protein alterations in PTEN, AR, MDM2 and p53 in canine prostate cancer (PC). Protein expression was evaluated by immunohistochemistry (15 normal, 22 proliferative inflammatory atrophy (PIA) and 19 PC samples) and Western blotting (2 normal prostate tissue, 2 BPH, 2 PIA samples and 2 PC samples) and gene expression by RT-qPCR (10 normal, 10 PIA and 15 PC samples) of formalin-fixed tissue. We identified nuclear and cytoplasmic expression of PTEN and p53 in all samples, with only nuclear staining found for MDM2 and AR. Our results revealed high expression of MDM2 in PC and PIA samples compared to normal samples, whereas PTEN, P53 and AR expression was down-regulated in PC compared to normal tissue. All tumour samples ($n = 19$) showed loss of nuclear PTEN expression, and all cancer mimickers showed positive nuclear staining. Therefore, nuclear PTEN staining could be a good diagnostic marker for differentiating between malignant lesions and mimickers. Canine prostate carcinogenesis involves increased expression of MDM2 in association with decreased expression of PTEN, p53 and AR, such as occurs in hormone refractory PC in men. Thus, dogs may be an important model for studying advanced stage PC.

1. Introduction

Dogs are commonly affected by proliferative prostatic lesions that are related to advanced age and changes in the levels of androgen hormones (Fonseca-Alves et al., 2013). Dogs are the only non-primate species that spontaneously and naturally develops prostate carcinoma (PC) (Palmieri et al., 2014). Dogs and humans have some similarities in the clinical and pathologic aspects of PC, including the presence of preneoplastic lesions and bone metastasis (Leroy et al., 2006). In fact, dogs are indicated as a potential animal model for studying the biological behaviour of various proliferative prostatic diseases that affect humans (Palmieri et al., 2014).

There is evidence that inflammation contributes to prostate carcinogenesis (Palapattu et al., 2004), causing repeated damage to the genome and resulting in increased cell proliferation (Montironi et al., 2007). Prostatic inflammatory atrophy (PIA) is a lesion that may have an inflammatory origin, and PIA has been identified in both humans and dogs (De Marzo et al., 2007; Fonseca-Alves et al., 2014; Palmieri et al., 2014; Fonseca-Alves et al., 2015). De Marzo et al. (2007) proposed that areas of PIA are direct precursors of prostatic intraepithelial neoplasia (PIN) or PC.

In humans, the risk of PC increases with the presence of mutated genes, such as *MDM2* and *AR* (Rubin and De Marzo, 2004), and tumour suppressor genes including phosphatase and tensin homolog (*PTEN*) and *TP53* play an important role in the origin of this neoplasia (Uzoh et al., 2009; Wozney and Antonarakis, 2014). PTEN acts as an antagonist of the Phosphatidylinositol-3 (PI3K) signalling pathway through the regulation of murine double minute 2 (MDM2) activity (Mayo and Donner, 2002). Under normal conditions, MDM2 inhibits the transcriptional activity of p53 and regulates cell cycle, DNA repair, senescence, apoptosis and angiogenesis (Pant and Lozano, 2014). The perturbation of these processes allows mutations to accumulate in a cell, which can be transferred to future generations, thereby increasing the risk of developing cancer (Barbieri and Tomlins, 2015).

MDM2 is overexpressed in various canine neoplasias, including osteosarcomas, mast cell tumours, soft tissue sarcomas and hepatoid gland

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tumours (Nakano et al., 2005; Wu et al., 2006). In human PC, MDM2 overexpression is correlated with a higher risk of metastasis, a reduced survival time for patients and increased hormonal refraction or resistance to antineoplastic agents (Khor et al., 2009; Yu et al., 2014). The close association between the PTEN-MDM2-p53 pathway and the androgen receptor (AR) enables normal prostate function (Carver et al., 2011; Wang et al., 2011). PTEN controls AR levels and is required for the maturation and differentiation of prostatic tissue (Nicholson and Ricke, 2011). However, in human PC, mutated PTEN confers a favourable environment for the activation of certain *anti*-apoptotic proteins, such as BCL-2 and BCL-XL, which have been implicated in the conversion of androgen-dependent PC to androgen-refractory PC (Sun et al., 2008).

In a previous study performed by our research group, we identified numerous copy number abnormalities in fourteen canine PC samples using comparative genomic hybridization (CGH), including copy number losses in the *TP53*, *PTEN* and *AR* genes and copy number gains in the *MDM2* gene (unpublished data). These previous results provided strong evidence that the PTEN-MDM2-p53 network is associated with the development of PC in dogs, but no previous studies have evaluated the protein or gene expression of factors in this network in canine PC.

The objectives of this study were to evaluate PTEN-MDM2-p53 and AR gene and protein expression in proliferative canine prostatic lesions and to compare these data with findings reported in humans.

2. Materials and methods

2.1. Samples

A total of 56 formalin-fixed, paraffin-embedded (FFPE) prostate tissues from dogs (aged 1–15 years, of pure or mixed breeds) were selected from the archives of the Veterinary Pathology Service, São Paulo State University (UNESP), Botucatu, SP, Brazil. The samples were collected during necropsy on animals that had an interval between death and necropsy of <24 h. All owners agreed to the necropsy.

Haematoxylin and eosin (H&E) blocks were cut for histopathological diagnosis, which was performed by three pathologists using a multihead microscope. The samples were classified as normal prostate, PIA or PC according to Palmieri et al. (2014) as part of the histopathological evaluation. From these slides, the most representative area for each diagnosis was selected and used to construct a TMA slide, as described by Bubendorf et al. (2001). The TMA slide consisted of 22 PIA, 19 PC and 15 normal prostate samples. Each lesion was collected from a different animal, and the sample tissues were placed on a TMA block in triplicate. After the TMA was constructed, the first and last slide sections were stained with H&E as an internal control for the TMA lesions.

We extracted mRNA from 35 FFPE samples, which were obtained from the archive (10 normal prostate, 10 PIA and 15 PC samples), not from the same animals used to generate the TMA. We used eight frozen prostatic tissue to western blotting analysis (2 normal prostate tissue, 2 BPH, 2 PIA samples and 2 PC samples).

2.2. Real-time reverse transcription PCR

The FFPE samples were manually macrodissected with needles (16 and 18 gauge) according to the procedure described by Pires et al. (2006), which enabled RNA extraction from only parts of tissue that met the pathological conditions under study. The processes of deparaffinization, protease and nuclease digestion, and nucleic acid isolation and purification were performed using a commercial RecoverAll™ Total Nucleic Acid Kit (Ambion, Life Technologies, MA, USA) according to the manufacturer's instructions. The RNA concentration was determined with a spectrophotometer (NanoDrop™, ND-8000, Thermo Scientific, MA, USA), while the RNA integrity was evaluated with a Bioanalyzer 2100 and an Agilent RNA 6000 Nano Kit (Agilent Technologies, CA, USA).

cDNA was synthesized in a final volume of 20 μL, and each reaction contained 1 μg of total RNA treated with DNAse I (Life Technologies, Rockville, MD, USA), 200 U of SuperScript III reverse transcriptase (Life Technologies), 4 μL of 5X SuperScript First-Strand Buffer, 1 μL of each dNTP at 10 mM (Life Technologies), 1 μL of Oligo-$(dT)_{18}$ (500 ng/μL) (Life Technologies), 1 μL of random hexamers (100 ng/μL) (Life Technologies), and 1 μL of 0.1 M DTT (Life Technologies). Reverse transcription was performed for 60 min at 50 °C, and the enzyme was subsequently inactivated for 15 min at 70 °C. cDNA was stored at −80 °C.

RT-qPCR for *PTEN, MDM2, TP53, AR* and the reference gene (Table 1) was conducted in a total volume of 10 μL containing Power SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA, USA), 1 μL of cDNA (1:10) and 0.3 μL of each primer. The reactions were performed in triplicate in 384-well plates using QuantStudio 12K Flex Thermal Cycler equipment (Applied Biosystems; Foster City, CA, USA). A dissociation curve was included in all experiments to determine the PCR product specificity. The most stable reference gene (*HPRT*) was chosen using geNorm software (Vandesompele et al., 2002) from a group of three genes (*GAPDH, HPRT*, and *RPL8*). Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.3. Western blotting

The blots were blocked with 3% bovine serum albumin in TBS-T (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 2 h and incubated overnight with the respective primary antibodies (PTEN — 1:1000; P53–1:500; AR — 1:250 and MDM2–1:1000). We used the goat *anti*-β-actin antibody (1:1000; sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA) such as control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, the blots were detected by means of chemiluminescence (Amersham ECL Select Western Blotting Detection Reagent, GE Helthcare). The protein expression was quantified by densitometric analysis of the bands and was expressed as integrated optical density (IOD). The protein expression was normalized to the β-actin values. Normalized data are expressed as the means ± SD.

2.4. Immunohistochemistry

The primary antibodies employed were validated in our laboratory for use in dogs in Western blot analysis with different canine prostatic tissues. The slides were deparaffinized in xylene and rehydrated in graded ethanol, and immunohistochemical staining was performed using a polymer system. Antigen retrieval was performed with citrate buffer (pH 6.0) in a pressure cooker (Pascal; Dako, Carpinteria, CA, USA), and no antigen retrieval was necessary for the PTEN antibody. Endogenous peroxidase activity was inhibited with 8% hydrogen peroxide in methanol for 20 min, and non-specific binding sites were blocked with 3% skim milk for 60 min at room temperature. The antibodies and dilutions are described in Table 2. A polymer system (Advance, Dako, Carpinteria, CA, USA) was applied as the secondary antibody. Peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (DAB+, Dako, Carpinteria, CA, USA), and the slides were counterstained with Harris haematoxylin. Negative controls were performed for all antibodies by replacing the primary antibody with immunoglobulin from the species in which the primary antibody was

Table 1
Primers sequences used for gene expression.

Gene	Foward	Reverse
PTEN	5′-CGACGGGAAGACAAGTTCATG-3′	5′-TCACCGCACACAGGCAAT-3′
MDM2	5′-GGGCCCCTTCGTGAGAATTG-3′	5′-GGTGTGGCTTTTCTCAGGGATT-3′
TP53	5′-GAACGCTGCTCTGACAGTAGTGA-3′	5′-CCCGCAAATTTCCTTCCA-3′
AR	5′-CGCCCCTGACCTGGTTT-3′	5′-GGCTGTACATCCGGGACTTG-3
HPRT	5′-AGCTTGCTGGTGAAAAGGAC-3′	5′-TTATAGTCAAGGGCATATCC-3′