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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

AVALIAÇÃO EPIGENÉTICA DOS GENES *NKX3.1* E *CDH1* E EXPRESSÃO DO C-MYC, NKX3.1 E E-CADERINA POR IMUNO-HISTOQUÍMICA EM MICROARRANJO DE TECIDO (TMA) DE LESÕES PRÉ-NEOPLÁSICAS E NEOPLÁSICAS NA PRÓSTATA DE CÃES

CARLOS EDUARDO FONSECA ALVES

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CARLOS EDUARDO FONSECA ALVES

Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

Orientadora: Profª Drª Renée Laufer Amorim
Co-Orientadora: Profª Drª Silvia Regina Rogatto
Co-Orientadora: Profª Drª Flavia Karina Delella

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COMISSÃO EXAMINADORA

Profª Drª Renée Laufer Amorim
Presidente e Orientadora
Departamento de Clínica Veterinária
FMVZ - UNESP - Botucatu

ProfªDrª Patricia Pintor Reis
Membro
Departamento de Cirurgia e Ortopedia
FMB – UNESP – Botucatu

Profª Drª Sandra Aparecida Drigo Linde
Membro
Departamento de Urologia
FMB – UNESP - Botucatu

Prof. Dr. Heidge Fukumasu
Membro
Departamento de Medicina Veterinária
FZA – USP – Pirassununga

Prof. Dr. Renato Lima Santos
Membro
Departamento de Clínica e Cirurgia Veterinárias
EV – UFMG – Belo Horizonte

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LISTA DE ABREVIATURAS

CP.....Carcinoma Prostático
FDA……..……*Food and Drug Administration*
VS.....Vesícula seminal
ZC.....Zona central
ZP.....Zona periférica
ZT.....Zona de transição
EFA.....Estromafibromuscular anterior
INCA.....Instituto Nacional do Câncer
PIN.....Neoplasia Intraepitelial Prostática
PIA.....Atrofia Inflamatória Proliferativa
HPB.....Hiperplasia Prostática Benigna
PSA.....Antígeno Prostático Canino

SUMÁRIO

FONSECA-ALVES, C.E. **Avaliação Epigenética dos Genes *NKX3.1* e *CDH1* e Expressão do C-MYC, NKX3.1 e E-Caderina por Imuno-histoquímica em Microarranjo de Tecido (TMA) de Lesões Pré-neoplásicas e Neoplásicas Na Próstata De Cães**. Botucatu, 2016. 123p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Botucatu, Universidade Estadual Paulista.

RESUMO

A próstata canina é um bom modelo para estudos comparados entre o cão e o homem, uma vez que essas duas espécies desenvolvem espontaneamente carcinoma de próstata (CP). Para melhor caracterização do CP canino, a presente pesquisa foi dividia em quatro capítulos que avaliam diferentes aspectos dos CPs em cães. A atrofia inflamatória proliferativa (PIA) é uma lesão pré-neoplásica descritas em humanos e pouco estuda em cães. Nós caracterizamos essa lesão em cães e identificamos uma forte relação entre a localização topográfica da PIA com os CPs. Além disso, foi identificada a perda de expressão gênica e proteica de PTEN e AR na PIA. Esses fatores associados corroboram com o potencial pré-neoplásico desta lesão em cães. Um achado interessante foi a alta expressão de P63 na PIA e em um grupo de CP caninos. Para melhor caracterizar este grupo, foi avaliada a expressão imuno-histoquímica de diferentes citoqueratinas e outras proteínas relacionadas ao desenvolvimento do CP em humanos. Os carcinomas que apresentam expressão de P63 apresentaram padrões morfológicos com escore de *Gleason* alto e um fenótipo mais agressivo quando comparado à tumores que não apresentação expressão de P63. Posteriormente, a expressão gênica e proteica de E-caderina, NKX3.1 e C-MYC foi avaliada em CP como marcadores nas diferentes lesões. Além disso, nós avaliamos a metilação como mecanismo regulatórios dos genes *CDH1* e *NKX3.1*. Foi possível identificar a perda de E-caderina e NKX3.1 nos tumores, comparado à próstata normal bem como o aumento da expressão de C-MYC. A expressão de E-caderina teve relação com a sobrevida dos pacientes e a hipermetilação do gene *CDH1* é o possível mecanismo regulatório deste gene. Não foram encontradas alterações de metilação no promotor do gene *NKX3.1*, indicando outro mecanismo na regulação deste gene.

Palavras-chave: canino, carcinoma prostático, metilação, mRNA, *Western blotting.*

FONSECA-ALVES, C.E. **Epigenetic evaluation of NKX3.1 and CDH1 and immunohistochemistry expression of C-MYC, NKX3.1 and E-cadherin using tissue microarray (TMA) of pre-neoplastic and neoplastic prostate of dogs**. Botucatu, 2016. 123p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

The canine prostate gland can be used as a model to human prostatic disease since dogs and men are the only species that spontaneously develop prostate carcinoma (PC). To better characterize the canine PC, this research was divided into four chapters that evaluated different aspects of the PC in dogs. The proliferative inflammatory atrophy (PIA) is a pre-neoplastic lesion described in humans and few studies in dogs describe it as a preneoplastic lesion. This study characterized PIA in dogs and identified a strong relationship between the PIA topography with PC. In addition, we identified the loss of PTEN and AR expression in PIA. These findings demonstrated the potential of PIA as a pre-neoplastic lesion in dogs. An interesting finding in this research was the high expression of P63 in PIA and a group of PC. This study found a group of PC showing P63 positive expression in neoplastic epithelial cells. Thus, these tumors were selected to better characterize them using immunohistochemistry. These tumors had an aggressive phenotype and presented high expression of AKT and C-MYC and loss of NKX3.1. Further, we selected a usual group of PC and evaluate the expression of E-cadherin, NKX3.1 and C-MYC. In addition, we evaluated the methylation as a regulatory mechanism of *CDH1* and *NKX3.1* genes. We have identified loss of E-cadherin and NKX3.1 in PC compared to normal prostate and C-MYC overexpression. The expression of E-cadherin was related to overall survival and Gleason score. The hypermethylation of CDH1can be the protein regulatory mechanism in canine PC. We did not find methylation alterations in *NKX3.1* promoter gene.

Keywords: canine, prostate cancer, methylation, mRNA, Western blotting.

CAPÍTULO I

1. INTRODUÇÃO

Segundo dados do Instituto Nacional do Câncer (INCA) para o ano de 2016 são esperados 68.800 novos casos de carcinoma prostático (CP), que é considerado como a segunda causa mais comum de mortes relacionadas ao câncer em homens com mais de 50 anos de idade. As estimativas mundiais apontam um crescimento em 25 vezes na incidência do CP, e este fato está associado ao diagnóstico precoce por meio do teste do Antígeno Prostático Específico (PSA) e o exame clínico de toque retal (INCA, 2016). Atualmente há uma grande discussão em torno do diagnóstico precoce do CP em humanos. As estatísticas apontam que 100% dos homens que chegarem aos 100 anos de idade, irão apresentar evidência histológicas de CP (INCA, 2016). No entanto, em 90% desses casos, os pacientes não irão apresentar progressão tumoral ou evolução clínica da doença (BANGMA et al., 2007). Assim, muitos pacientes idosos que apresentarem tumores indolentes podem passar por procedimento invasivos de forma desnecessária.

Na medicina veterinária, os dados relacionados à incidência das neoplasias prostáticas em cães são controversos. No entanto, há um consenso ao afirmar que em cães, o CP apresenta incidência menor que no homem. As afecções prostáticas benignas são frequentemente encontradas na clínica dos animais de companhia, especialmente em cães. As alterações da próstata mais comuns são a hiperplasia prostática benigna (HPB), a prostatite (FONSECA-ALVES et al., 2010) e os cistos prostáticos (FONSECA-ALVES et al., 2012). De acordo com JOHNSTON et al. (2000), 100% dos cães não castrados apresentaram alterações histológicas prostáticas com o avançar da idade. Em cães, não é reportada alta incidência de CP indolente. De acordo com a literatura veterinária, os CP são agressivos, altamente metastáticos e apresentam prognóstico desfavorável (LEROY & NORTHRUP, 2009). A próstata canina é considerada modelo para estudo comparativo da próstata humana, uma vez que essas são as únicas duas espécies em que ocorre espontaneamente o câncer prostático, neoplasia intraepitelial prostática (PIN), hiperplasia prostática benigna (HPB) (BOSTWICK et al., 2000) e a atrofia inflamatória proliferativa (PIA) (PALMIERI et al., 2014). O CP canino compartilha muitas características semelhantes ao do homem, e seria um

modelo de estudo para o CP invasivo/metastático (BELL et al., 1991; BOSTWICK & QIAN, 2004).

Na medicina humana, a imunomarcação de proteínas específicas para as células basais é utilizada para diferenciar lesões pré-neoplásicas dos carcinomas prostáticos (TAN et al., 2015). Dentre as principais lesões pré-neoplásicas descritas na medicina humana, destacam-se a PIN (ZHOU et al., 2003) e a PIA (DE MARZO et al., 1999). Em cães, existe uma controvérsia em relação a alta frequência e influência da PIN no desenvolvimento do CP e a literatura sobre a relação da PIA e os CP é escassa (PALMIERI et al., 2014). WATERS & BOSTWICK (1997) e BOSTIWICK et al. (2000) encontraram uma alta incidência de PIN em cães (mais de 80% das amostras analisadas), no entanto, os autores avaliaram um número muito pequeno de amostras. MADEWELL et al. (2004) e AQUILINA et al. (1998) realizaram dois grandes estudos avaliando a frequência da PIN em amostras de tecido prostático canino e encontraram frequência de PIN menor que 3% no tecido prostático canino. Um estudo brasileiro (MATSUZAKI et al., 2010) identificou uma baixa frequência da PIN em lesões prostáticas caninas e em todos os casos, tratava-se de lesões de baixo grau (*low grade* PIN).

Em CP humanos, a perda da expressão das proteínas E-caderina e NKX3.1 é amplamente descrita na em tumores com alto escore de *Gleason* (DEBELEC-BUTUNER et al., 2014). A perda do NKX3.1 e E-caderina se correlaciona com a alta incidência de metástases dos pacientes. Outro gene importante para os CP em homens é a expressão do gene *C-MYC*. O gene *C-MYC* codifica a proteína MYC que apresenta papel de regulação da proliferação celular em condições fisiológicas (FARIA & RABENHORST, 2006). No CP em homens, a oncoproteína MYC apresenta expressão aumentada e, os níveis de transcritos do gene *C-MYC* se correlaciona com o desenvolvimento de metástases nos pacientes (ANDERSON et al., 2012). Em cães, apenas um estudo avaliou a expressão de NKX3.1 e MYC em CP caninos (FONSECA-ALVES et al., 2013).

Devido ao limitado conhecimento sobre a biologia dos CP em cães, a presenta pesquisa realizou um estudo morfológico, imuno-histoquímico e molecular para caracterização de diferentes lesões pré-neoplásicas e neoplásicas da próstata de cães.

2. REVISÃO DE LITERATURA

Anatomia e Histologia comparada

A próstata, um órgão essencialmente hormônio-dependente, é parcialmente músculo-glandular, com ductos que se abrem no segmento prostático da uretra. Em cães, consiste em dois lobos laterais envoltos em uma cápsula fibrosa (Figura 1) contendo fibras musculares lisas (FONSECA-ALVES et al., 2010). Compreende a única glândula sexual anexa dos cães e sua principal função é a produção do fluido prostático, que transporta e sustenta os espermatozoides durante o processo de ejaculação (JONES et al., 2000).

Em homens, a glândula prostática é localizada no compartimento subperitoneal entre o diafragma pélvico e a cavidade peritoneal (LEE et al., 2011). Apresenta localização posterior à sínfise púbica, anterior ao reto e inferior à bexiga urinária (LEE et al., 2011). A glândula prostática apresenta formato cônico (em forma de noz) e circunda a uretra prostática (LEE et al., 2011). Anatomicamente a próstata humana é dividida em zonas (Figura 2) devido as particularidades histológicas de cada zona. Nas duas espécies, devido à proximidade com o reto, é possível realizar palpação digital para diagnóstico de afecções nesta glândula.

Figura 1. Corte transversal na região medial da glândula prostática canina. Note a presenta de dois lobos laterais e a presença da uretra na região medial da glândula prostática.

Fonte: Setor de patologia – FMVZ, UNESP, campus de Botucatu

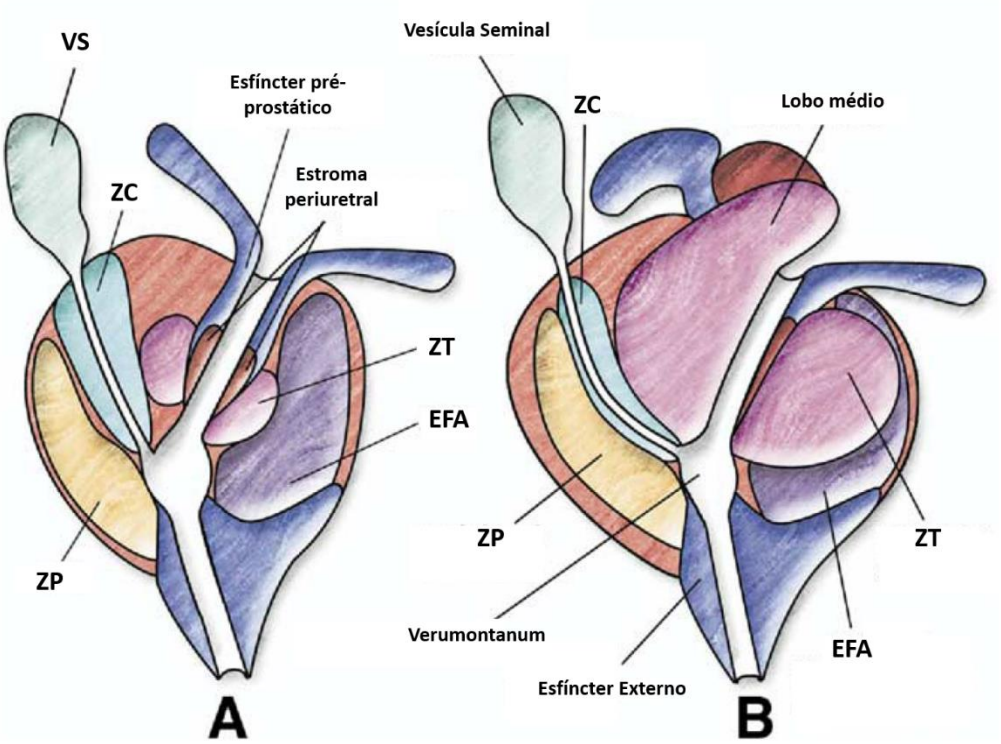

Figura 2. Desenho esquemático da anatomia prostática humana. VS: vesícula seminal; ZC: zona central; ZP: zona periférica; ZT: zona de transição; EFA: estroma fibromuscular anterior.
Fonte: MCLAUGHLINET et al. (2005).

Histologicamente, a próstata é formada por lóbulos e estes de ácinos glandulares sustentados por um estroma de tecido conjuntivo e musculatura lisa, envoltos por uma cápsula fibrosa muscular espessa (COTRAN et al., 2000). O epitélio glandular colunar muda para transicional nos ductos excretores que se abrem no interior da uretra. No parênquima prostático as células são de dois tipos: epiteliais e estromais. As células epiteliais são divididas em dois principais grupos: células luminais, que são colunares altas, com função secretória e as células basais, que são precursoras de epitélio secretório. As células estromais compreendem os fibroblastos e células musculares lisas, vasos sanguíneos e nervos (BARSANTI & FINCO, 1992).

Hiperplasia Prostática Benigna (HPB)

A hiperplasia prostática benigna (HPB) ocorre em cães adultos e idosos, sendo considerada a alteração mais comum da próstata canina. Compreende aumento progressivo e induzido por hormônios, sendo que 100% dos cães adultos não castrados desenvolvem evidências histológicas de hiperplasia com o avançar da idade (JOHNSTON et al., 2000).

As espécies canina e humana, com o processo de senescência, desenvolvem espontaneamente HPB. No homem, é um processo nodular que se origina na zona de transição, ocorrendo hiperplasia e proliferação estromal em direção a uretra (VICHERAT, et al., 2003). Em contraste, no cão a HPB é um processo difuso em que há hiperplasia do epitélio secretório de ácinos pré-existentes (LOWSETH et al., 1990).

As hiperplasias podem ser classificadas em epitelial cística, epitelial papilífera e estromal, destacando que, em alguns casos, pode ocorrer associação entre essas. Para a classificação da hiperplasia estromal considera-se a proliferação do estroma fibroso ou muscular, associada, com frequência, à atrofia glandular e infiltrado inflamatório mononuclear. A forma epitelial cística ocorre quando o epitélio glandular cúbico se apresenta hiperplásico e com formação de grandes cavidades císticas. Já a hiperplasia epitelial papilífera caracteriza-se por múltiplas projeções digitiformes do epitélio glandular ao lúmen, com aumento do número de camadas de células secretoras (FONSECA-ALVES et al., 2010).

Neoplasia Prostática

Nos seres humanos e cães, o adenocarcinoma prostático é a neoplasia prostática mais comum. Em ambas as espécies acomete indivíduos de meia idade a idosos, sendo que nos cães parece haver uma ocorrência maior de casos em animais de médio à grande porte (SWINNEY, 1998). Alguns autores sugerem que a grande maioria dos casos ocorre em cães a partir de 8 anos de idade, com média de 8,9 anos (KRAWIECK & HEFLIN, 1992). Na espécie humana, a neoplasia prostática também é considerada uma doença senil, sendo responsável por mais de 50% de mortes associadas ao câncer em homens acima de 70 anos (ARMBRUSTER, 1993). A similaridade na patogenia do câncer prostático entre cães e humanos têm tornado a espécie canina

modelo natural para estudo dessa afecção, apesar de neoplasias prostáticas não serem tão comuns em cães como o são no homem, mas é uma lesão extremamente rara em qualquer outra espécie animal (LOWSETH et al., 1990; KARR et al., 1995).

Segundo dados do Instituto Nacional do Câncer (INCA) para o ano de 2016 foram esperados 68.800 novos casos de CP em homens sendo a neoplasia mais comum para indivíduos do sexo masculino excluindo os tumores cutâneos não melanoma. Após o início do uso do antígeno prostático especifico (PSA) na rotina clínica urológica para a detecção do carcinoma prostático, houve um aumento crescente do número de casos diagnosticados precocemente (ETZIONI et al., 2002). No Estados Unidos (EUA), no início da década de 80 os bancos de dados registravam uma incidência de 86 casos para cada 100.000 homens, com início do uso do PSA na rotina clínica esse número aumento para 179 casos para 100.000 habitantes no início da década de 90 (BANGMA et al., 2007). A avaliação sérica do PSA passou a ser amplamente utilizada a partir de 1986, quando o *Food and Drug Administration* (FDA) aprovou seu uso, no entanto, a partir disso, poucos estudos foram realizados para verificar a real importância do teste para o diagnóstico precoce em homens (ETZIONI et al., 2002). Atualmente vários pesquisadores questionam a validade do PSA para a detecção precoce de neoplasias prostáticas, considerando que muitos tumores diagnosticados não acarretarão em nenhuma alteração clínica para o paciente, constituindo apenas em um achado de biópsia (SOUNG, 2015; BOKHORST et al., 2015).

Em cães, um estudo retrospectivo desenvolvido na Universidade do Missouri identificou uma incidência dos carcinomas prostáticos de 71,6% dentre os tumores do trato genital masculino e uma média de 39,7 novos casos por ano (BRYAN et al., 2007). Não há dados epidemiológicas em relação a frequência do CP nos cães em outros países. No entanto, nos últimos anos, houve um aumento no número de publicações envolvendo neoplasias prostáticas caninas, apesar da incidência desta neoplasia ser considerada baixa (PALMIERI et al., 2014; LEAN et al., 2014; SHAFIEE et al., 2014). A baixa incidência relatada por alguns autores, pode se relacionar aos fatores ambientais locais que poderiam aumentar a incidência de CaP na população canina (FONSECA-ALVES, 2013). Um estudo anterior realizado por nosso

grupo de pesquisa, avaliando amostras prostáticas caninas, relatou uma alta taxa de carcinomas prostáticos na cidade de Goiânia, com uma incidência estimada de 19,1% das amostras avaliadas (FONSECA-ALVES, 2013). Realizando uma busca na base de dados do INCA, é possível verificar que em Goiânia há alta incidência de CP em homens, sendo a cidade brasileira com maior número de casos por 100.000 habitantes, igualando aos índices de alguns países desenvolvidos (INCA, 2016).

Recentemente, uma pesquisa realizou coleta de amostras prostáticas de cães necropsiados no serviço de patologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista e encontramos um alto índice de neoplasias prostáticas em cães sem nenhuma alteração clínica (Figura 3) (Dados não publicados). Além do CP constituir um achado de necropsia, foi verificado a presença de êmbolos metastáticos positivos para o PSA em alguns animais. Com essas informações, acreditamos que como no homem, o cão apresenta CPs com comportamento biológico variável, podendo ocorrer como uma doença insidiosa ou evoluir para doença metastática causando óbito do animal.

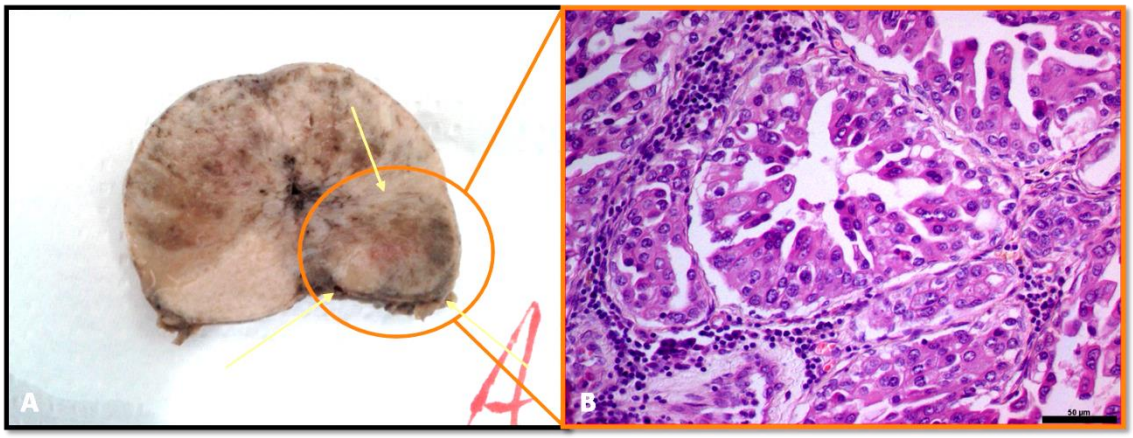

Figura 3. A: Avaliação macroscópica de uma glândula prostática canina, evidenciando região nodular, de aspecto acastanhado, circunscrito, em um cão sem sinais clínicos de doença prostática. B: Avaliação histopatológica da região evidenciada na figura 3A, revelando proliferação neoplásica das células epiteliais prostáticas, com arquitetura cribiforme, associada a um alto pleomorfismo celular. 20x.

O CP nos cães compartilha várias características com o de humanos, como a morfologia, presença concomitante de focos de PIN, PIA e metástases ósseas (TESKE et al., 2002; PALMIERI et al., 2014). Na espécie canina, os sinais clínicos frequentemente encontrados são tenesmo e disúria, podendo ocorrer hematúria, anorexia e perda de peso (CORNELL et al., 2000). Os distúrbios de micção são amplamente descritos no homem e no cão, e se relacionam a infiltração neoplásica da uretra (Figura 4) (LEAN et al., 2014). O prognóstico do paciente acometido pelo carcinoma prostático é desfavorável, pois ao diagnóstico os cães apresentam um quadro avançado da doença, com infiltração de estruturas adjacentes e metástases à distância (FONSECA-ALVES et al., 2015).

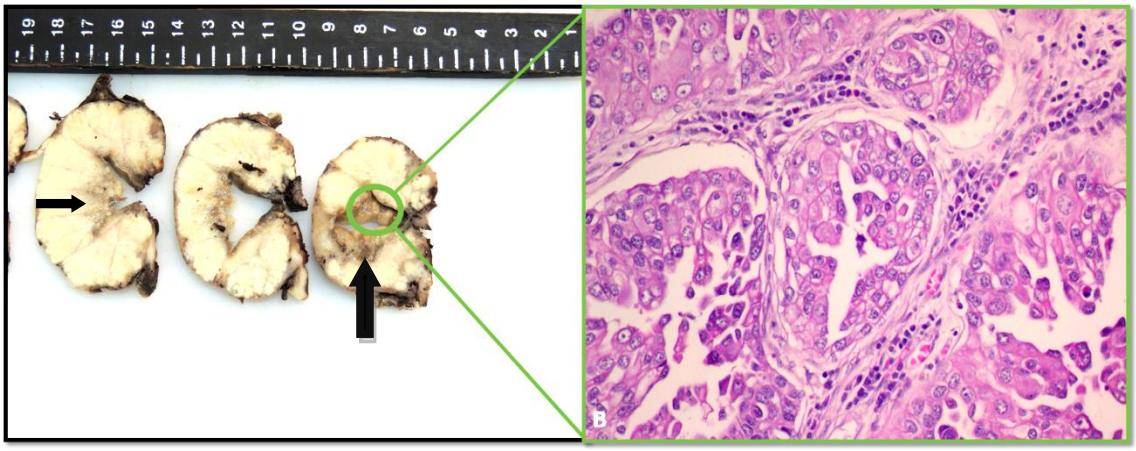

Figura 4. A: aspecto macroscópico após cortes sagitais no parênquima prostático de um cão com suspeita de neoplasia prostática. Note a dilatação da uretra com infiltração neoplásica destruindo o parênquima (setas). B: Avaliação histopatológica de lesão prostática peri-uretral revelando proliferação neoplásica de células epiteliais, com padrão acinar. Nota-se intenso pleomorfismo celular e infiltrado inflamatório associado.

Escore de *Gleason*

O escore de *Gleason* é uma graduação histopatológica das neoplasias prostáticas em humanos que pontua de 1 até 5 a arquitetura dos tumores prostáticos. Tumores diferenciados com arquitetura acinar recebem o escore “1”, enquanto tumores pouco diferenciados com perda total da arquitetura acinar recebem o escore “5”. O escore final é o resultado de uma soma em pares gerando um escore final de 2 até 10. Na atualidade, somente lesões com escore de *Gleason* de no mínimo 6 (3+3) são consideradas para o diagnóstico histopatológico definitivo. Ao receber um escore menor que 6, a lesão não é considerada um carcinoma, e sim, uma lesão benigna que mimetiza o CP (MIAH et al., 2016).

Os critérios estabelecidos pelo Dr. Gleason associados ao exame de PSA sérico e o toque retal são as três principais ferramentas necessárias para estabelecer um protocolo terapêutico para um paciente. Os escore de *Gleason* em humanos já foi modificado três vezes para se adequar as evoluções no diagnóstico dos CPs em humanos (MIAH et al., 2016).

O escore de *Gleason* em cães foi adaptado recentemente da literatura humana (Figura 5), através de um sistema de graduação semelhante ao do homem, em escores 1, 2 e 3 que representam os tumores com morfologia mais próxima dos ácinos prostáticos normais, e escores 4 e 5 em que se perde a arquitetura acinar (PALMIERI; GRIECO,2015)

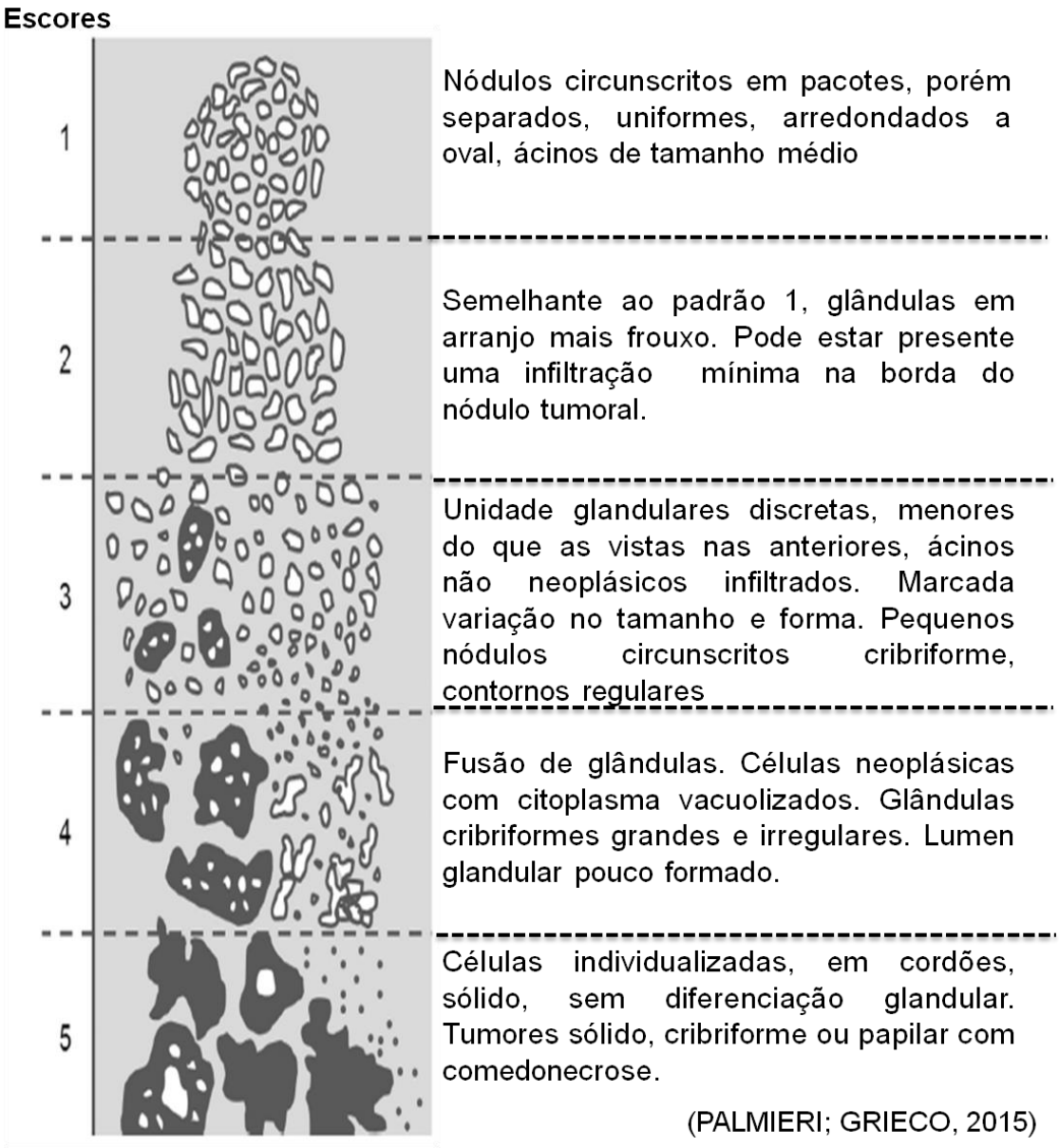

Figura 5. Esquema representando os critérios de *Gleason* para a classificação dos carcinomas prostáticos. O sistema de pontuação varia de 1 até 5.
Fonte: KOBAYASHI (2016).

Gene *C-MYC*

O gene *C-MYC* é considerado um oncogene e apresenta papel central no processo carcinogênico em diversas neoplasias humanas (FRANK et al., 2001). A proteína MYC apresenta participação direta e indireta na regulação do crescimento celular, instabilidade genômica, diferenciação, metabolismo, ciclo celular, apoptose, imortalidade e angiogênese (FARIA & RABENHORST, 2006). A presença de amplificações do gene *C-MYC* foi associada com o processo metastático em CP humanos (LIU et al., 2004). Novas pesquisas direcionadas para identificação dos

genes-alvo e elucidar os mecanismos moleculares regulados pelo *C-MYC* contribuíram substancialmente para o entendimento do CP no homem (FRANK et al., 2001, LIU et al., 2004). O maior desafio, todavia, consiste no desenvolvimento de ferramentas capazes de modular a interação entre a proteína MYC e os seus alvos moleculares, desvendando potenciais estratégias terapêuticas (MADY et al., 2001).

O *C-MYC* é um proto-oncogene localizado no cromossomo 8 (8q23-24) no homem, que atua de forma complexa na carcinogênese humana, presente nos processos de proliferação, crescimento celular e na apoptose (GARTE, 1993; LIU et al., 2004). Na regulação do ciclo celular, a proteína C-MYC é necessária para progressão das células primárias da fase G1 para fase S (TELEPIS et al., 2003). Dentre os mecanismos descritos em diferentes tumores, destacam-se as translocações cromossômicas, como verificado no linfoma de Burkitt, através da justaposição da região promotora do gene de cadeia pesada de imunoglobulina, altamente expressa em células B, ao lado do sítio gênico do *C-MYC*; amplificação gênica, pelo aumento do número de cópias e, consequentemente, de sua expressão; estímulo da transcrição gênica, como observado nos carcinomas do cólon; inserção de retrovírus adjacente ao gene *C-MYC*, ativando sua expressão via sequências regulatórias virais, entre outros (RYAN & BIRNIE, 1996; GARTE, 1993; LIU et al., 2004).

Os progressos obtidos no entendimento da regulação do gene *C-MYC* e na identificação dos seus "genes-alvo" evocam também a possibilidade da elaboração de estratégias direcionadas à manipulação das vias reguladas por esse gene em células neoplásicas (EISENMAN, 2001). Manobras moleculares realizadas in vitro, como o uso de RNA *antisense* em gliomas, já demonstraram a capacidade de inibição da expressão do *C-MYC*, suprimindo a atividade proliferativa dos tumores (BROADDUS et al., 1997). Outras vias passíveis de intervenção consistem nos diferentes níveis de regulação dos mecanismos de transcrição, tradução e ativação transcricional da oncoproteína C-MYC, bem como sua interação com o DNA e com outras proteínas reguladoras associadas (LUSCHER & EISENMAN, 1990). Demonstrou-se que a baixa expressão de C-MYC é acompanhada de diferenciação precoce e parada permanente do ciclo celular (CANELLES et al., 1997). Por outro lado, a alta expressão de MYC é suficiente para induzir diferentes mecanismos de diferenciação celular (ONCLERCQ et al., 1999; FARIA & RABENHORST, 2006).

Em cães, o gene *C-MYC* está localizado no cromossomo 15 e apenas um trabalho demonstrou alterações na expressão proteica de MYC em CP caninos (FONSECA-ALVES et al., 2013). Em amostras de próstata normais, foi descrita a expressão citoplasma fraca de MYC e a translocação nuclear da proteína nos CP

caninos (FONSECA-ALVES et al., 2013). Não há nenhum estudo na literatura investigando os mecanismos moleculares envolvidos na desregulação do gene C-MYC em neoplasias prostáticas caninas.

Gene *NKX3.1*

Os mecanismos envolvidos na carcinogênese e no desenvolvimento embrionário são semelhantes, já que ambos os processos são dependentes (de forma diferente) da proliferação e diferenciação celular (GRIER et al., 2005). Os genes do desenvolvimento embrionário, como os da família *homeobox*, aparecem como alvos importantes no estudo do câncer. Os genes membros da subfamília NK *homeobox* participam da organogênese e fisiologia de alguns tecidos, principalmente no sistema urogenital (ABATE-SHEN et al., 2003).

O *NKX3.1* é um gene supressor tumoral, localizado no *locus* cromossômico 8p21.2 em humanos. A expressão desta proteína ocorre principalmente nas células epiteliais do lúmen prostático (GRIER et al., 2005). A proteína NKX3.1 possui estrutura tridimensional preservada graças aos aminoácidos que se mantiveram conservados durante a evolução (GELMANN et al., 2002). Há intima relação entre a expressão de *NKX3.1* e a regulação hormonal. Sabe-se que esse gene está intimamente relacionado com a expressão androgênica, fato este se relaciona a ausência de expressão de NKX3.1 em animais isogênicos castrados (SCIAVOLINO et al., 1997).

Nos estágios iniciais da embriogênese, o esclerótomo e as células musculares lisas do endotélio vascular expressam NKX3.1. Nas fases finais do desenvolvimento embrionário, a proteína NKX3.1 é expressa especificadamente em células epiteliais prostáticas (GELMANN et al., 2002; SONG et al., 2009). A expressão de NKX3.1 é importante para desenvolvimento e funcionamento fisiológico da glândula prostática em homens (SONG et al., 2009). Alterações neste gene induzem defeitos na morfogênese ductal, na secreção de proteínas e contribui para carcinogênese prostática (KORKMAZ et al., 2004; ZHENG et al., 2006; ZHANG et al., 2008).

Um dos mecanismos implicados no silenciamento do gene NKX3.1 é a hipermetilação da região promotora deste gene (LI et al., 2002). Em modelos de camundongos *knockouts* para o gene NKX3.1, foi verificado alterações na formação dos ductos prostáticos e foi relata a atrofia do epitélio prostático (STEADMAN et al., 2002). Os animais *knockouts* para o gene NKX3.1 desenvolvem lesões pré-neoplásicas (PIA). No entanto somente a perda da expressão deste gene não é suficiente para induzir o CP *in* vivo (STEADMAN et al., 2002). Em homens, 80% dos tumores prostáticos com alto escore de *Gleason* apresentam diminuição da expressão

desse *NKX3.1*. No entanto, a perda desta proteína não ocorre em tumores de baixo grau (que são a maioria dos tumores em humanos) (ZHANG et al., 2008).

E-caderina

O gene *CDH1* humano está localizado no braço longo do cromossomo 16 (16q22.1), sendo responsável pela codificação da proteína E-caderina. A proteína E-caderina é membro da família das caderinas, molécula de adesão intercelular dependente de cálcio (WATABE et al., 1994), localizada na superfície basolateral das células epiteliais. O gene da E-caderina possui uma sequência codificadora que dá origem a um peptídeo de sinal de 27 aminoácidos (éxons 1 e 2), um peptídeo precursor de154 aminoácidos (éxons 2 a 4) e uma proteína madura de 728 aminoácidos (COMIJN et al., 2001).

A E-caderina tem um papel importante na determinação de um fenótipo do tecido epitelial, sendo responsável pela regulação da migração e diferenciação celular (REIS-FILHO et al., 2003). No processo carcinogênese dos tecidos epiteliais, a ausência de E-caderina está relacionada com a invasão em tecidos adjacentes e com processo de metástase (REIS-FILHO et al., 2003). O papel da E-caderina na progressão tumoral tem sido extensivamente estudado nos últimos anos; os resultados disponíveis sugerem uma correlação entre a perda ou redução da E-caderina e a progressão neoplásica (PALACIOS et al., 1995).

Algumas neoplasias em humanos já foram relacionadas à perda completa ou parcial da expressão da E-caderina, dentre elas, carcinomas de pâncreas, mama, rim, fígado, estômago, tireoide, cólon, ovário e próstata (SAHA, et al., 2008), ocorrendo relação direta entre a diminuição da expressão e perda da diferenciação e menor sobrevida dos pacientes, sendo consideração assim por estes autores como potencial marcador prognóstico nestes tumores.

3. Objetivos

Objetivo Geral

- Caracterizar as lesões prostáticas caninas pré-neoplásicas e neoplásicas usando avaliação morfológica, imuno-histoquímica e técnicas moleculares.

Objetivos Específicos

- Caracterizar a atrofia inflamatória proliferativa (PIA) na próstata canina
- Caracterizar pela técnica de imuno-histoquímica um grupo de tumores caninos que apresentam expressão da proteína P63
- Avaliar a expressão de E-caderina nas neoplasias prostáticas caninas e investigar os mecanismos envolvendo a regulação deste gene no carcinoma prostático
- Avaliar a expressão gênica e proteica de NKX3.1 e C-MYC no carcinoma prostático canino

CAPÍTULO II

ARTIGO CIENTÍFICO 1

TRABALHO CIENTÍFICO A SER SUBMETIDO PARA A REVISTA "BJU INTERNATIONAL"

http://www.bjuinternational.com/

Implication of proliferative inflammatory atrophy in canine prostate carcinogenesis

Objective

To evaluate the topographic relation among PIA, PIN and PC lesions and the cytokeratin immunophenotype of PIA lesions in canine prostate. Additionally, we evaluated P53, MDM2, AR and PTEN protein and gene expression in PIA lesions, compared to normal prostates, to better characterize its preneoplastic potential in dogs.

Patients and Methods

Four hundred sixth nine hematoxylin (H&E) and eosin slides from canine prostatic tissue were selected from our Veterinary Pathology archive and we diagnosed 50 normal prostates, 140 benign prostatic hyperplasia, 171 proliferative inflammatory atrophy (PIA), 84 prostate cancer (PC), 14 prostatic intraepithelial neoplasia (PIN) and 10 bacterial prostatitis. After diagnosis, classified canine PIA according to human classification and the PC H&E slides to evaluate the presence of PIA and PIN lesions surrounding the neoplastic tissue. From the H&E slides, we selected 40 formalin-fixed paraffin-embedded (FFPE) (20 normal prostates and 20 PIA lesions) to evaluateP53, MDM2, AR and PTEN gene and protein expression. Additionally, proliferative index was evaluated, using Ki67 immunostaining.

Results

All PIA lesions (171) showed some degree of mononuclear cells around the glands. All samples with PIN diagnosis (14/14) had adjacent PIA lesion. We did not find any PC samples showing adjacent PIN foci. 60% (51/84) of the PC samples had adjacent PIA lesions and we found an evidence of histological transition among benign tissue (normal or hyperplastic), PIA and PC in 21.5% (11/51) of the slides. Normal prostatic tissue was positive for PSA (100% - 20/20) and all samples (20/20) had score 4 for Pan-cytokeratin expression. In normal prostatic tissue, HMWC and P63 were present in the basal cells, showing a discontinuous layer (100% - 20/20). In all PIA samples, it was possible to observe positive staining for PSA and for pancytokeratin (score 4) (100% - 20/20). Interestingly, PIA samples showed a continuous basal cell layer positive for P63and HMWC. Thus, the epithelial glands from PIA had an intermediate phenotype (PSA+/P63+/HMWC+). We identified a decreased in PTEN and AR expression in PIA samples compared to normal samples.

The normal prostatic tissue had no Ki-67 expression in epithelial luminal cells. It was possible to observe only few basal Ki-67 positive cells. Normal prostatic tissue had a mean of 2.2 (±1.5) Ki-67 positive cells and PIA lesions showed a mean of 45.1 (31.8). In PIA, the luminal and basal cells showed a higher number of Ki67 positive cells than normal prostate ($P<0.0001$).

Conclusions

We identified PIA simple atrophy as a common lesion in dog prostate and this lesion shares many morphological similarities with human PIA. The high proliferative index and the downregulation of PTEN and AR in PIA lesion can reflect its preneoplastic potential.

Keywords

Dog, Prostatic Atrophy, Preneoplastic lesion.

Introduction

There are many evidences that inflammation may contribute to prostate carcinogenesis [1], causing repeated damage to the genome, resulting in increased cell proliferation [2]. The inflammatory process in the prostate gland is associated with epithelium atrophy, high proliferative index and decreased expression of apoptotic markers [1,2]. De Marzo et al. [3] proposed the term PIA to designate the discrete focus of glandular epithelium proliferation with morphological appearance of simple atrophy associated with different degrees of inflammation.

In men, PIA lesion occurs in the peripheral zone of the prostate gland, where is observed most frequently prostate cancer (PC) [3].The mononuclear inflammatory infiltrate is frequently associated with PIA lesions [3,4].And it occurs in the peripheral area of human prostate [5]. The presence of these inflammatory cells in prostatic microenvironment leads to the secretion of proteases, mitogenic, antiapoptotic and angiogenic factors [6]; that induce epithelial cell atrophy in a first stage and then cell proliferation [4].

PIA occurs adjacent to high-grade prostatic intraepithelial neoplasia (HGPIN) and prostate cancer (PC) and has similar genetic abnormalities [7,8]. The most accepted theory indicates a progression from PIA to HGPIN and subsequently to PC [8,9]. A previous study had characterize the morphology and immunophenotype of PIA lesions and found 34% focal atrophic lesions surrounding HGPIN lesions indicating a morphology progression from PIA to HGPIN [3].Few studies had shown the presence of PIA in canine prostate [10,11,12,13]. However, these studies evaluated only morphological and

immunohistochemical characteristics of PIA lesions and did not evaluate its preneoplastic potential.

Chromosomal imbalances were previously described in canine PIA lesions [14].Copy number gain was detected in *CRYGS, ADIPOQ, SST* genes and copy number losses were identified in *CD38, ZNF518B, WDR1, SLC2A9* genes [14]. These results showed the chromosomal instability in PIA lesions from dogs and represent a strong evidence of the preneoplastic potential of PIA lesions. In addition, the loss of E-cadherin in PIA lesions has been reporter in Veterinary literature [13]. Prostatic epithelial cells can lose E-cadherin during cell division and re-expression after it completes [15]. This alteration can be explained by DNA hypermethylation in the promoter region of E-cadherin gene (unpublished results). Thus, all these previous research had indicated very complex cytogenetic and epigenetic imbalances in canine PIA.

Our study aimed to establish a topographic relation between PIA, PIN and PC lesions and evaluate the cytokeratin immunophenotype of PIA lesions in canine prostate. Additionally, we evaluated TP53, MDM2, AR and PTEN protein and gene expression in PIA lesions, compared to normal prostates, to better characterize its preneoplastic potential in dogs.

Material and Methods

Tissue selection

Four hundred sixth nine hematoxylin and eosin (H&E) slides were selected from our Veterinary Pathology archive (Univ. Estadual Paulista-UNESP) with prostatic lesion diagnosis. The histopathological slides were reevaluated according to Fonseca-Alves et al. [13] for normal prostate, BPH and prostatitis; Palmieri et al. [12] for PIN and PC. To characterize PIA lesions,

we adapted the human histological classification [3]. All samples were from adult intact dogs. We diagnosed 50 normal prostates, 140 benign prostatic hyperplasia (BPH), 171 proliferative inflammatory atrophy (PIA), 84 prostate carcinoma (PC), 14 prostatic intraepithelial neoplasia (PIN) and 10 bacterial prostatitis. Each diagnosis represented a different patient.

After diagnosis, we used PIA slides to apply the modified human classification and the PC H&E slides to evaluate the presence of pre neoplastic lesions (PIA and PIN)surrounding the neoplastic tissue. From the H&E slides, we selected 40 formalin-fixed paraffin-embedded (FFPE) (20 normal prostates and 20 PIA lesions) to evaluateP53, MDM2, AR and PTEN gene and protein expression.

Scoring of Morphological Features

The intensity of inflammation was adapted from De Marzo et al. [3]. Briefly, all H&E PIA slides were evaluated using a numerical scale from 0 up to 6, which 0 represents no inflammation, 1/2 represents low inflammation, 3/4 moderate inflammation and 5/6 intense inflammation. Epithelial appearance of the atrophic glands was evaluated according to Marzo et al. [3] with adaptations. The key findings to identify PIA lesion in a low power field were glandular overall hyperchromatic appearance, inflammatory infiltrate, loss of papillary architecture and cuboidal cell morphology. In a higher power filed the key findings were acini showing at least two epithelial cell layers, atrophic appearance of cuboidal cells with scant cytoplasm, presence of evident nucleolus and mitotic figures.

Lesions were divided into simple atrophy, post-atrophic hyperplasia (PAH) and mixed pattern (simple atrophy and PAH), according to De Marzo et al [3]. We also evaluated the presence of dilated glands as referred as cystic atrophy.

Immunohistochemistry

Our research group previously validated the antibody cross reactivity against canine prostatic tissue [16]. Slide sections were dewaxed in xylene and rehydrated in graded ethanol. For antigen retrieval, the slides were incubated with citrate buffer (pH 6.0) in a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA). The slides were treated with freshly prepared 3% hydrogen peroxide in methanol for 20 min and further washed in Tris-buffered saline. The primary antibodies were diluted in 1:500 for PTEN (Bioss, Massachusetts, USA - Bs-0686R), 1:50 for MDM2 (Abcam, Cambridge, UK - ab38618), 1:50 for P53 (Santa Cruz Biotecnology,sc-75366), 1:100 for AR (Abcam, Cambridge, UK - ab77557), 1:2000 for PSA (Bioss, polyclonal), 1:300 for high molecular weight cytokeratin (HMWC) (DakoCytomation, clone: 34βE12), 1:100 for P63 (DakoCytomation, clone 4A4), 1:300 for Pan-cytokeratin (Invitrogen), 1:50 for Ki-67 (DakoCytomation, clone: MIB1)and were incubated over night at 4°C. A polymer system (Envision, Dako, Carpinteria, CA, USA) was applied as a secondary antibody conjugated to peroxidase and 3´-diaminobenzidine tetrahydrochloride (DAB1, Dako, Carpinteria, CA, USA) was used as the chromogen, for 5 min, followed by Harris hematoxylin counterstain.

After each step of the immunohistochemical process, the slides were rinsed with Tris-buffered saline. Negative controls were performed for all

antibodies by omitting the primary antibody and replacing them with Tris-buffered saline. Normal prostate was used as positive control for all antibodies.

Scoring Immunohistochemical Features

The samples were scored based on an assessment of the percentage of positive for each antibody (P53, MDM2, AR, PTEN, P63, HMWC and Pan-cytokeratin). From 1 to 10% of positive cells was scored 0, 11 to 25% positive cells was scored 1; 26 to 50% positive cells was scored 2; 51 to 75% positive cells was scored 3; and more than 75% positive cells was scored 4. Three collaborators (CEFA, PEK and RLA) independently interpreted the immunohistochemical results. PSA antibody was recorded as positive or negative staining by the epithelial cells. We also evaluated the P63, HMWC and pancyokeratin immunolocalization (basal or luminal cells). Ki-67 evaluation was made by counting the number of positive cells per total of cells, according to De Marzo et al. [3].

Gene expression

All FFPE samples were macrodissected using 16 gauge needles and mRNA were extracted using a commercial RecoverAll™ Total Nucleic Acid Kit (Ambion, Life Technologies, MA, USA) according to the manufacturer's instructions. The mRNA concentration was determined with a spectrophotometer (NanoDrop™, ND-8000, Thermo Scientific, MA, USA), while the mRNA 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 50ºC, and the enzyme was subsequently inactivated for 15 min at 70ºC. cDNA was stored at -80ºC.

The *AR*, *TP53*, *MDM2*, *PTEN* and the endogenous genes (Table 1) were 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. Relative gene expression was quantified using the 2-ΔΔCT method [17].

Data analysis

Statistical analyses were performed using GraphPad Prism v.5.0 (GraphPad Software Inc., La Jolla, CA, USA). Kruskal-Wallis or Mann-Whitney U tests was applied to compare *TP53*, *MDM2*, *AR* and *PTEN* transcription levels between normal and PIA samples. The chi-square exact test was used to evaluate difference immunohistochemical expression between normal and PIA samples.

Results

Morphological Features

All PIA lesions (171) showed some degree of mononuclear cells around the glands. In 35.5% (61/171) of PIA samples, was possible to observe low inflammatory infiltrate (Figure 1). Moderate inflammation it was observed in 42.1% (72/171) of PIA samples (Figure 2) and intense inflammatory infiltrate in 22.4% (38/171) (Figure 3).

Simple atrophy was the most common histopathological feature. We observed simple atrophy in 73% of all cases (125/171), mixed pattern was observed in 17.5% (30/171) and PAH was observed in 9,5% (16/171).

All samples with PIN diagnosis (14/14) had adjacent PIA lesion. We did not find any PC samples showing adjacent PIN foci. 60% (51/84) of the PC samples had adjacent PIA lesions and we found an evidence of histological transition among benign tissue (normal or hyperplastic), PIA and PC in 21.5% (11/51) (Figure 4). The other 40% (33/84) of the canine PC showed only neoplastic tissue in H&E slides.

Immunohistochemical Features

The Immunohistochemical results are presented in table 2. The P53 antibody showed both nuclear and cytoplasmic staining (Figure 5). 45% (9/20) of prostatic tissue samples showed score 4 and 55% (11/20) showed score 3 of P53 immunoexpression. Regarding PIA samples, 40% (8/20) showed score 4, 40%% (8/20) showed score 3 and 20% (4/20) score 2. There was no statistical difference in P53 scores between normal and PIA samples.

MDM2 protein showed nuclear expression. One normal samples (5%) was score 4. 30% (6/20) were score 3 and 65% (13/20) score 2. 15% (3/20) of PIA samples showed score 4, 30% (6/20) showed score 3 and 55% (11/20) showed score 2. There was no statistical difference of MDM2 expression in normal and PIA samples.

We identified a decreased PTEN expression in PIA samples compared to normal samples (P=0.003). All normal samples showed more than 75% of cells positive (score 4) against PTEN. Two PIA samples had score 4, 25% (5/20) score 3, 25% (5/20) score 2 and the other eight PIA showed score 1.

All normal prostate samples (20/20) showed more than 75% of AR positive. We identified a decreased expression (p=0.01) in PIA samples compared to normal samples. 55% (11/20) of PIA samples showed score 3 and 45% (9/20) had score 2. All immunohistochemical results are shown in table 2.

Normal prostatic tissue was positive for PSA (100% - 20/20) (Figure 5A) and all samples (20/20) had score 4 for Pan-cytokeratin expression. In normal prostatic tissue HMWC and P63 was present in the basal cells, showing a discontinuous layer (100% - 20/20). In all PIA samples, it was possible to observe positive staining for PSA and fro Pan-cytokeratin (score 4) (100% - 20/20). Interestingly, PIA samples showed a continuous basal cell layer positive for P63 (Figure 5B) and HMWC (Figure 5C); all samples being score 4 for P63 and HMWC. Thus, the epithelial glands from PIA had an intermediate phenotype (PSA+/P63+/HMWC+).

The normal prostatic tissue had no Ki-67 expression in epithelial luminal cells. It was possible to observe only few basal Ki-67 positive cell. Normal prostatic tissue had a mean of 2.2±1.5Ki-67 positive cells and PIA lesions

showed a mean of 45.1±31.8) Ki-67 positive cells. In PIA, the luminal and basal cells showed a higher number of Ki67 positive cells than normal prostate (P<0.0001).

RT-qPCR results

There was no difference in TP53 (Figure 6A) and MDM2 (Figure 6B) transcript levels between normal and PIA samples (P > 0.05). We identified a positive correlation between TP53 and MDM2 transcript levels in normal (R=0.7754; P<0.0001) and PIA (R=0.6573; P= 0.0202) samples. Thus, in normal (Figure 6C) and PIA (Figure 6D) samples the increased TP53 transcript level was correlated with a concomitant increase.

It was possible to observe a decreased PTEN (P=0.307) and AR (P=0.0008) expression in PIA compared to normal samples. The median of relative quantification (RQ) of *AR* in normal samples was 1.8 (0.3-9). PIA samples showed a median RQ of 0.7±0.1-1.6(Figure 6E). The median of *PTEN* RQ (Figure 6F) was 1.4±0.3 - 6.7 and 0.7±0.2 - 3 in normal and PIA samples respectively.

Discussion

Our results indicate that PIA lesion is a very common change in canine prostate in intact dogs. In South America and Europe, is less frequent to spay male dogs. Thus, these countries have a unique opportunity to better understand the role of PIA as a preneoplastic lesion in dogs.

We found PIA lesion in 36.5% of the canine prostatic tissues and PIN samples represented 3% (14/469). The veterinary literature brings a controversial incidence of PIN lesions in canine prostate. Previously, Waters et al. [18] and Bostwick et al. [19] found a higher incidence of PIN in canine

prostatic tissue from dogs. However, two larger studies in dogs evaluate the frequency of PIN lesion in canine prostatic tissue, showing no histological evidence of PC [20,21].These studies had shown a very low frequency (less than 3%) of PIN lesions in canine prostatic tissue[20,21].

We found PIA lesions surrounding canine PC in 60% of PC cases and in 21.5% of these cases we identified an evident transition among, benign tissue, PIA lesions and invasive prostate cancer. This result represents an evidence of PIA as a pre-neoplastic lesion in canine prostate. Furthermore, we found a higher proliferative index (Ki-67 expression) in PIA lesions compared to normal prostate, suggesting a higher proliferative potential of PIA lesions.

We did not find any correlation between P53 and MDM2 protein expression and PIA. However, there was a correlation between*TP53* and *MDM2* transcript levels in normal prostates and PIA. *TP53* and *MDM2* expression were widely studied in human cancers and *MDM2* is a negative regulator of *TP53* transcript [22,23,24]. The positive correlation between *TP53*/*MDM2* indicates that samples with higher *TP53* transcripts had higher *MDM2* levels. This correlation can indicate a role of *MDM2*in controlling*TP53* transcript, as in humans [23]. In canine PC, the upregulation of *MDM2* and downregulation of *TP53* transcript levels were previously described [16]. Nevertheless, our results did not indicate alterations in *TP53* and *MDM2* in PIA.

Our results showed a downregulation of *AR* gene and protein in PIA samples compared to normal prostate. *AR* gene has a key role in prostatic development and maintenance [25]. In dogs, downregulation of *AR* genes common in PC development [25]. Previously, our research group found *AR* copy number loss in canine PC (unpublished data). Thus, the *AR*

downregulation in PIA lesions associated with the highest proliferative index can indicate an independence of AR stimulation to prostatic cellular proliferation. Furthermore, we found PTEN gene and protein downregulation in PIA samples. In human PC, PTEN loss was correlated with PC androgen independence [26]. PTEN downregulation can be associated with the decreased AR levels and these alterations are associated with activation of anti-apoptotic pathway [26,27].

Canine PC shows a heterogeneous pattern of cytokeratin expression and there is a consensus about the intermediate phenotype (luminal markers+/basal markers+) of canine PC [11,28,29,30]. To the best of our knowledge, there are no previous papers showing canine PIA cytokeratin pattern. As in human PIA, we identified an enrichment of intermediate cells in PIA lesion.

Conclusions

We identified simple atrophy as a common lesion in dog prostate and shares many morphological similarities with human PIA. The high proliferative index and the downregulation of PTEN and AR in PIA lesions can reflect their preneoplastic potential. Canine PIA lesions shows an intermediate(PSA+/P63+/HMWC+)phenotype and these cells could be cancer progenitor cells in canine PC.

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Table 1. Forward and Reverse primer sequence for each gene used in RT-qPCR analyzes.

Gene symbol	Location	Primer sequences
AR	Chromosome 24	F: 5'-CGCCCCTGACCTGGTTT-3' R: 5'-GGCTGTACATCCGGGACTTG-3'
PTEN	Chromosome 26	F: 5'-CGACGGGAAGACAAGTTCATG-3' R: 5'-TCACCGCACACAGGCAAT-3'
MDM2	Chromosome 10	F : 5'-GGGCCCCTTCGTGAGAATTG-3' R: 5'-GGTGTGGCTTTTCTCAGGGATT-3'
TP53	Chromosome 5	F: 5'-GAACGCTGCTCTGACAGTAGTGA-3' R: 5'-CCCGCAAATTTCCTTCCA-3'
HPRT	Chromosome X	F: 5'-AGCTTGCTGGTGAAAAGGAC-3' R: 5'-TTATAGTCAAGGGCATATCC-3'

F: forward; R: reverse

Table 2: PTEN, P53, MDM2 and AR Immunohistochemical expression in canine normal prostate and PIA samples.

		Score				
	Group	**1**	**2**	**3**	**4**	P
PTEN	Normal	0% (0/20)	0% (0/20)	0% (0/20)	100% (20/20)	P=0.003
	PIA	40% (8/20)	25% (5/20)	25% (5/20)	10% (2/20)	
P53	Normal	0% (0/20)	0% (0/20)	55% (11/20)	45% (9/20)	P>0.05
	PIA	0% (0/20)	20% (4/20)	40% (8/20)	40% (8/20)	
MDM2	Normal	0% (0/20)	65% (13/20)	30% (6/20)	5% (1/20)	P>0.05
	PIA	0% (0/20)	55% (11/20)	30% (6/20)	15% (3/20)	
AR	Normal	0% (0/20)	0% (0/20)	0% (0/20)	100% (20/20)	P=0.01
	PIA	0% (0/20)	45% (9/20)	55% (11/20)	0% (0/20)	

From 11 to 25% of positive cells was scored 1; 26 to 50% positive cells was scored 2; 51 to 75% positive cells was scored 3; and more than 75% positive cells was scored 4

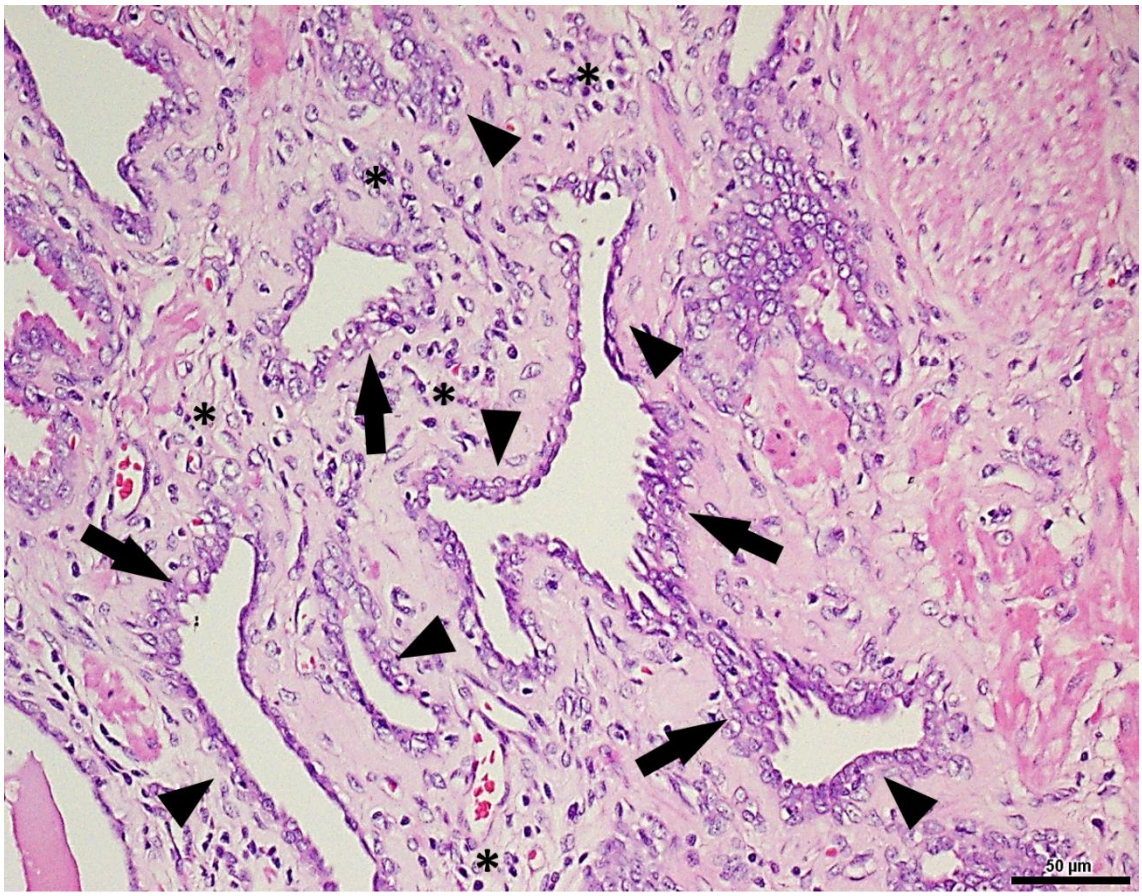

Figure 1. Hematoxylin and eosin (H&E) staining of a canine prostate, showing proliferative inflammatory atrophy (PIA). It is possible to note discrete mononuclear inflammatory infiltrate (asterisk) and areas of prostatic gland atrophy with cells showing hyperchromatic nuclei and at least one layer (arrowhead). Note areas with low-grade prostatic intraepithelial neoplasia (LGPIN) (arrows).Bar = 50µm

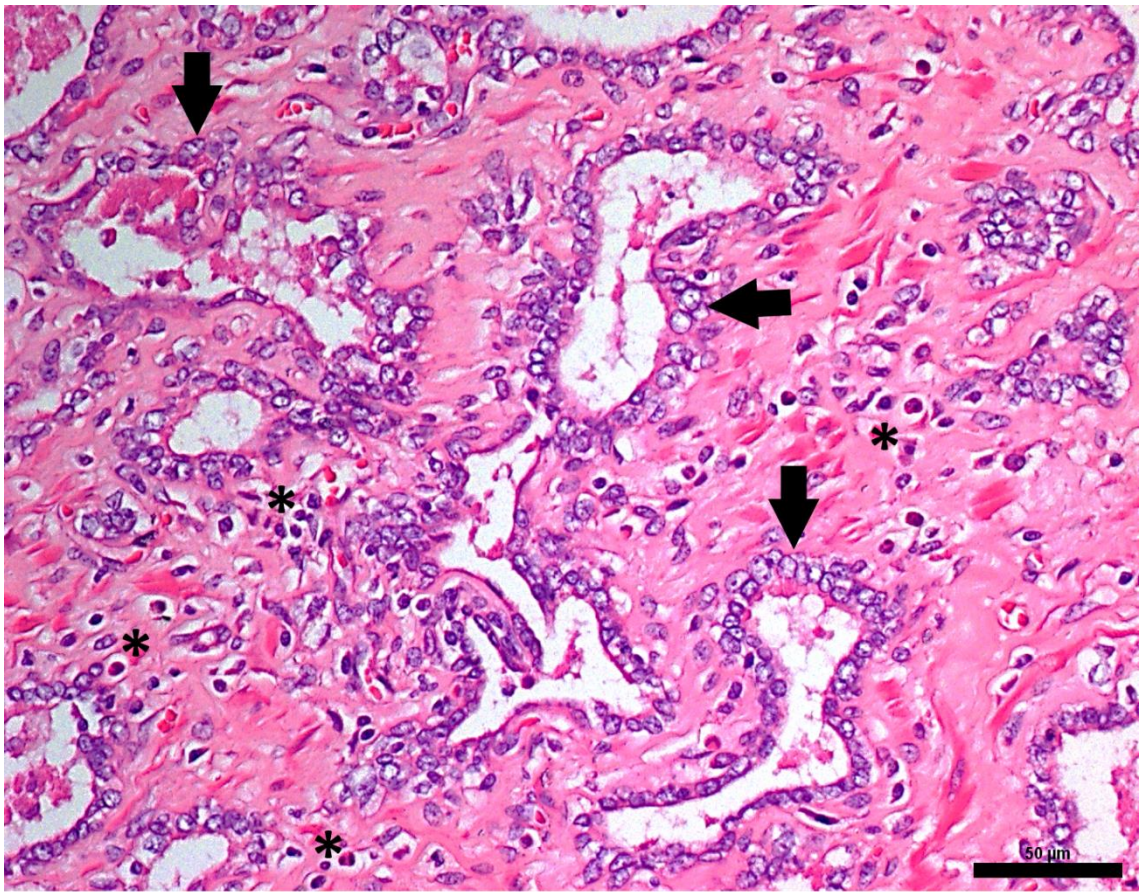

Figure 2. Hematoxylin and eosin (H&E) staining of a canine prostate with proliferative inflammatory atrophy (PIA). It is possible to note moderate inflammatory infiltrate (asterisk) and atrophic epithelial showing at least one layer and evident nuclei (arrow). Bar = 50µm.

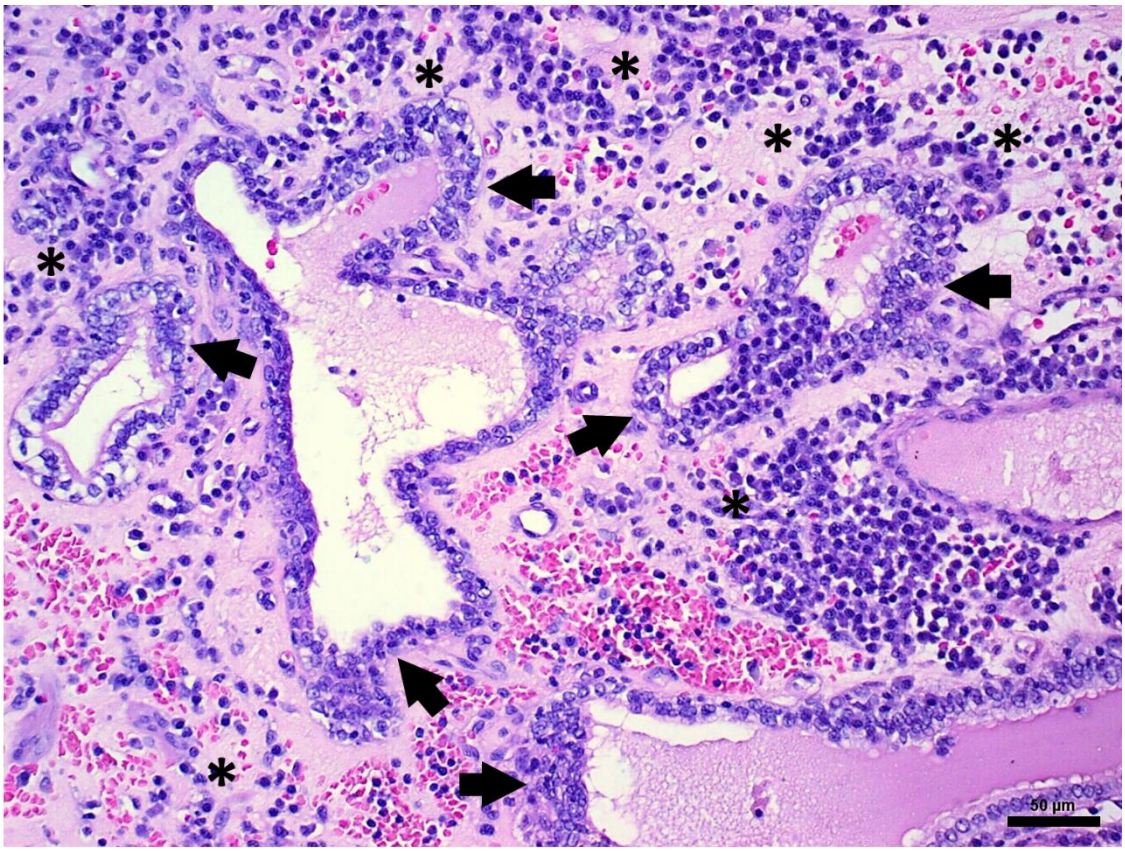

Figure 3. Hematoxylin and eosin (H&E) staining of a canine prostate. It is possible to note an intense mononuclear inflammatory infiltrate (asterisk) with atrophic epithelial cells showing at least two layers (arrows), hyperchromatic nuclei and evident nucleolus. Bar = 50µm

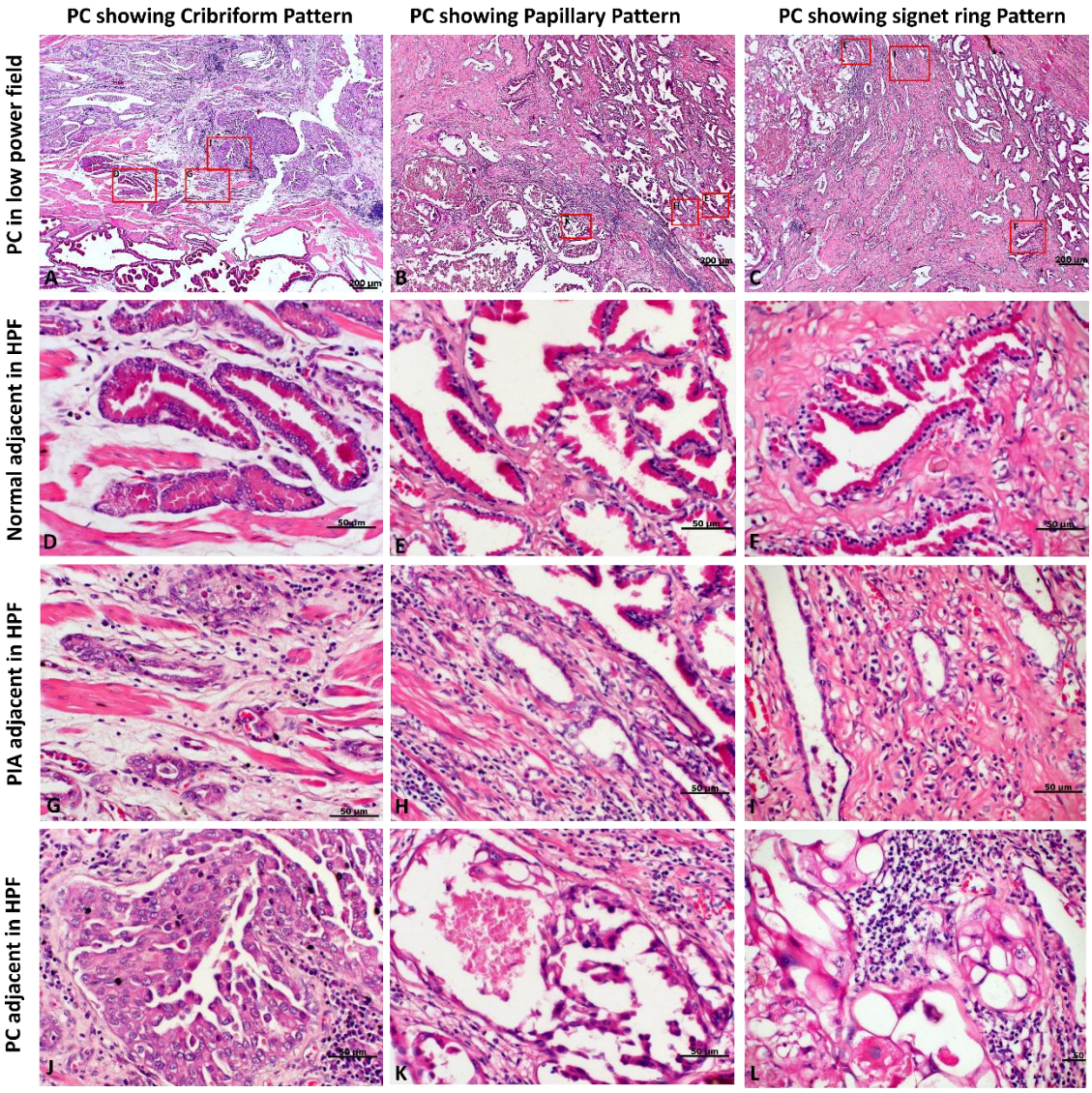

Figure 4. Hematoxylin and eosin (H&E) staining from three different canine prostate carcinomas (PC). A, B and C: Note in a low power field a carcinoma area surrounding by a proliferative inflammatory atrophy and benign adjacent area. D, E and F: high power field (insert of figures A, B and C respectively) of adjacent normal tissue. It is possible to note the epithelial cell layer with a columnar epithelium showing no histological changes. G, H and I: High power field of surrounding proliferative inflammatory atrophy (PIA) (the areas from the red rectangles in figures A, B and C. It is possible to note the atrophic glands showing at least one layer and hyperchromatic nuclei. J, K, L: high power field of a carcinoma area. Note a cribriform (J), papillary (K) and signet ring (L) pattern of the PC.

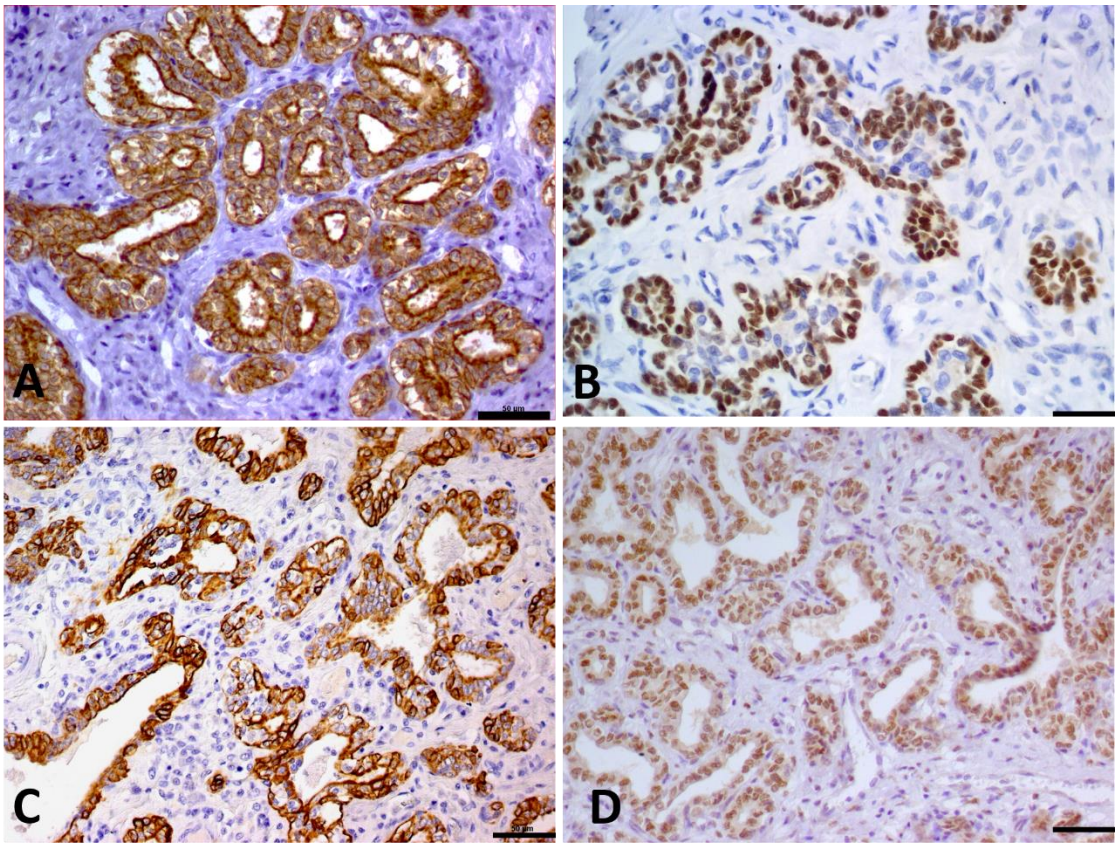

Figure 5. Immunohistochemistry staining of proliferative inflammatory atrophy (PIA) of the canine prostate. A: Prostatic specific antigen (PSA) in a PIA lesion. It was possible to note a strong cytoplasmic expression of atrophic epithelial cells. B: P63 staining in PIA. It was possible to observe nuclear staining in both basal and epithelial cells. In basal cell layer, it was possible to note a continuous p63 positive staining. C: High molecular weight cytokeratin staining of PIA. It was possible to note membranous staining in both epithelial and basal cells. D: AR expression in canine PIA. Note nuclear staining in luminal cells.

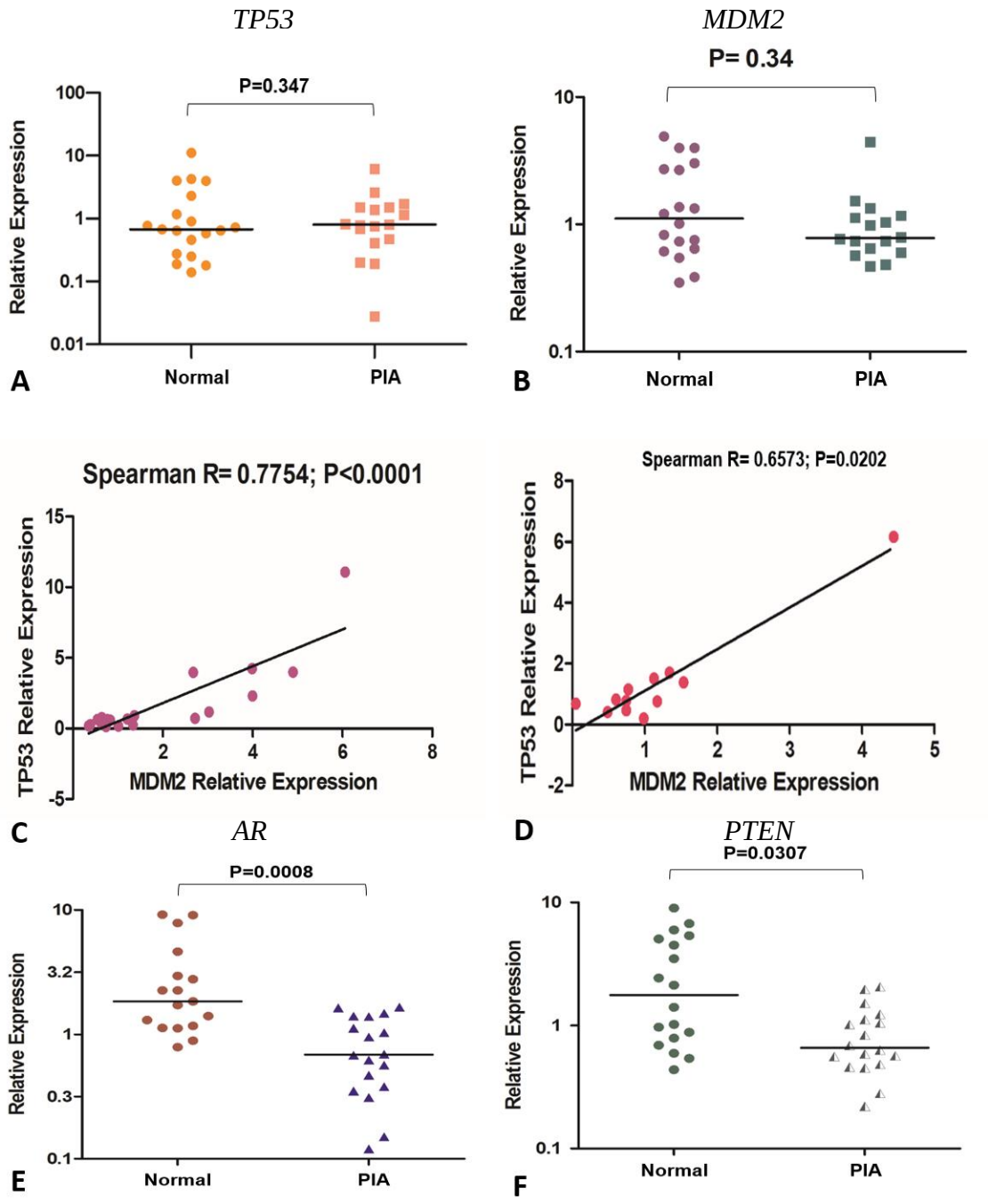

Figure 6. A: *TP53* transcript levels in normal and proliferative inflammatory atrophy (PIA) of canine prostate. Normal and PIA had similar median of expression. B: *MDM2* transcript levels in normal and PIA samples. There was no statistical difference of *MDM2* expression between normal and PIA samples. C and D: correlation between *TP53* and *MDM2* transcript levels in normal and PIA samples respectively. Note a positive correlation in both analyses. E: *AR* transcript levels in normal and PIA samples. PIA samples had a lower median of expression compared to normal tissue. E: *PTEN* expression in normal and PIA samples. PIA had a lower transcript levels compared to normal tissue.

CAPÍTULO III

ARTIGO CIENTÍFICO 2

TRABALHO CIENTÍFICO SUBMETIDO PARA REVISTA "HISTOCHEMISTRY AND CELL BIOLOGY"

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Immunohistochemical characterization of a canine prostate carcinoma cohort showing aberrant p63 expression

Abstract

This study aimed to investigate several molecular markers to better characterize a cohort of prostate cancer (PC) aberrantly expressing p63, and compare them with p63- PC and normal canine prostatic tissue, as well as with the human p63+ PC. Twenty formalin-fixed, paraffin-embedded canine PC samples were selected according to p63 expression (10 p63+ PC and 10 p63- PC) and compared with 10 normal prostatic tissues. The p63 expression patterns were confirmed by immunohistochemistry and Western blotting assays and were correlated with the expression of high molecular weight cytokeratin (HMWC), CK8/18, CK5, AR, PSA, Chromogranin, NKX3.1, PTEN, AKT and C-MYC by immunohistochemistry. CD44+/CD24-cancer stem cell markers were also assessed. The p63+ canine PC samples were also positive for CK5, HMWC and CK8/18. Interestingly, in normal tissues, basal cells were arranged in a discontinuous manner, whereas tumor sections showed a continuous basal cells layer. Canine p63+ PC samples showed cytoplasmic staining to all basal markers and were negative for PSA, NKX3.1, PTEN and chromogranin. Four of these samples were negative for AR (4/10), and the remaining samples had low AR expression (6/10). Both p63+ and p63- tumor samples showed higher cytoplasmic AKT expression and nuclear C-MYC staining. All PC cells were of the CD44+/CD24- phenotype. In contrast to human PC, canine PC that aberrantly expressed p63 showed high expression of HMWC and CK5 and loss of NKX3.1. These findings may contribute to understanding the cellular origin of PC and to better characterize canine PC.

Key words: dog, prostate cancer, comparative oncology, luminal cells, basal cells.

1. Introduction

Canine prostatic disorders are very common in small animal medicine, and benign prostatic hyperplasia is the most common diagnosis in dogs (Teske et al. 2002; Alves et al. 2014). Canine prostate cancer (PC) represents 13% of all prostatic disorders, and the very aggressive behavior is associated with a high metastasis rate and poor prognosis (Fonseca-Alves et al. 2015). Canine PC has been proposed as a comparative oncology model to better understand human tumors (Teske et al. 2002; Alves et al. 2014). Dogs and humans share many particularities concerning prostate disorders, such as the development of age-related benign prostatic hyperplasia (BPH), proliferative inflammatory atrophy (PIA), prostatic intraepithelial neoplasia (PIN) and PC (Fonseca-Alves et al. 2015; Palmieri et al. 2014). Nevertheless, there are differences between canine prostate tissue and the human prostatic gland (Alves et al. 2014; Leav et al. 2001), such as a discontinuous basal cell layer and fewer basal cells in normal canine prostatic tissues (Leav et al. 2001; Romanucci et al. 2014). Canine PC characterization is the first step to demonstrate the utility of dogs as a model for human PC studies and to assess the potential role of this model in the design of therapeutic targets (Fonseca-Alves et al. 2014).

Basal cells are implicated in prostate development, and the basal membrane plays a pivotal role in supporting stromal and epithelial tissues (Bostwick et al. 1987). In epithelial carcinogenesis, loss of basal integrity is an important event for invasion and metastasis (Baydar et al. 2011). Expression patterns of high-molecular weight (HMW/34βE12) cytokeratin and p63 in basal cells are widely used as diagnostic markers for human prostate cancer (Baydar et al. 2011; Lawson et al. 2010). The transcription factor p63 belongs to the p53 family and is expressed in the nuclei of basal cells (Tan et al. 2015). Human normal prostatic tissue shows a continuous basal cell layer that is positive for p63. In contrast, during carcinogenesis, the basal cell layer is lost, and human PC lacks p63-positive cells (Bostwick et al. 1987; Tan et al. 2015). Based on these findings, p63 has been used for diagnostic purposes to differentiate PC from mimickers (Tan et al. 2015). In contrast, basal cell markers are not used for diagnosis of PC in veterinary medicine (Fonseca-Alves et al. 2013). Romanucci et al. (2014) reported the loss of p63 expression in canine PC; however, one carcinoma was described to have aberrant p63 expression. Previously, we reported the aberrant expression of p63 and HMWC in canine PC (Romanucci et al.

2014). These tumors also showed a continuous basal cell layer, which was different from the discontinuous basal cell layer observed in normal canine prostate tissue (Romanucci et al. 2014).

Although a correlation between basal cells and the development of human PC has been proposed, the origin of prostatic carcinoma is still under debate (Collins et al. 2005; Wang et al. 2009; Goldstein et al. 2010). It is widely accepted that luminal cells are the cellular origin of human prostate cancer, due to the loss of basal cells during carcinogenesis [14]. However, recent studies have proposed that PC could originate from three different epithelial prostatic cells: luminal cells (CK5-/CK8+), intermediate cells (CK5+/CK8+) and basal cells (CK5+/CK8-) [10,14,16].

Interestingly, a group of rare human prostate adenocarcinomas showing aberrant p63 expression in luminal neoplastic cells was recently described (Tan et al. 2015; Dhillon et al. 2009; Zhou et al. 2003; Osunkoya et al. 2008). These tumors appear to be molecularly distinct from the usual type of prostate cancer (Tan et al. 2015). Due to the recent descriptions of human and canine prostate cancer with aberrant p63 expression and the importance of these tumors in both species, the aim of this study was characterize a group of canine p63+ PC, comparing them with canine p63- PC, normal prostatic tissue and the human p63+ aberrantly expressing tumors. First, the basal and luminal markers were evaluated in the groups of tumors, and than some proteins related to prognosis were also evaluated, to get information about the aggressiveness of the p63 abnormally expressing canine prostatic tumors.

MATERIALS AND METHODS

Case Selection

Seventy-four formalin-fixed, paraffin-embedded (FFPE) canine PC samples and 28 fresh frozen tissues collected from 2011-2015 were retrieved from the archives of the Veterinary Pathology Service, Veterinary School, UNESP, SP-Brazil. In addition, 10 FFPE normal prostatic gland tissues were obtained. All the cases were evaluated for p63 protein expression by immunohistochemistry using an automated system (Autostainer Classic, Dako Cytomation). Based on p63 expression patterns, 20 PC cases were selected, 10 with aberrant p63 expression (p63+) and 10 with no p63 expression (p63-). For the Western blot analysis, we selected seven normal prostate tissues and seven p63+ PC tissues (fresh frozen tissues). Tumor samples were obtained during prostatectomy surgery in animals with PC. Normal tissues were collected during necropsy from animals that died from causes not related to prostatic disease, and the interval between death and necropsy was less than 30 minutes.

Medical records were reviewed to obtain clinical information, treatment modalities, treatment response and outcome. Inclusion criteria specified animals with a computed tomography (CT), abdominal ultrasound and/or thoracic X-ray and histopathological analysis. Radical prostatectomy was the primary treatment for animals with non-metastatic PC (3/20). Four patients (4/20) received metronomic chemotherapy, four (4/20) received only piroxicam, five (5/20) did not receive any treatment, and there was no clinical information for the remaining four (4/20). Metronomic chemotherapy was provided according to Fonseca-Alves et al. (2015). Unfortunately, outcome information was unavailable for nine patients (9/20).

Histopathological Analysis

Histopathological diagnosis was established according to Lai et al. (2008), and the Gleason-like score was determined according to Palmieri and Grieco (2015).

Protein Expression Analysis by Immunohistochemistry

Slide sections were dewaxed in xylene and rehydrated in graded ethanol. For antigen retrieval, the slides were incubated with citrate buffer (pH 6.0) for 30 seconds in a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA). The slides were treated with freshly prepared 3% hydrogen peroxide in methanol for 20 min to inhibit endogenous peroxidase activity and then washed in Tris-buffered saline.

The primary antibodies with their respective dilutions are shown in Supplementary Table 1. A peroxidase-conjugated polymer system (Envision, Dako, Carpinteria, CA, USA) was utilized as a secondary antibody; the slides were incubated with the chromogen 3´-diaminobenzidine tetrahydrochloride (DAB1, Dako, Carpinteria, CA, USA) for 5 min and then counterstained with Harris hematoxylin.

After each step of the immunohistochemical process, the slides were rinsed with Tris-buffered saline. Negative controls were performed for all antibodies by replacing the primary antibody with Tris-buffered saline. Canine normal prostate tissue was used as a positive control for CK8/18, CK5, p63 and 34βE12 (HMWC), AR, PSA, NKX3.1, AKT, C-MYC and PTEN expression. Normal canine pancreas was used as a positive control for Chromogranin expression. Canine mammary gland carcinoma tissue known to be positive for CD44 and CD24 was used as a positive control for these markers.

The protein expression of p63, HMWC, Ki67, CK8/18, CK5, CD44, CD24, PTEN, AKT, C-MYC, Chromogranin and NKX3.1 was evaluated according to Wang et al. (2014) with modifications. Briefly, the slides were graded according to staining intensity and the number of positive cells: score 4, intense positive staining in more than 75% of target cells; score 3, strong positive staining in 50-75% of cells; score 2, intense staining in 30-50% of cells or less intense staining in greater than 50% of cells; and score 1, intense staining in more than 5% but less than 30% of cells or less intense staining in more than 10% but less than 50% of cells. Lesser degrees of staining were designated as indeterminate (or ambiguous), and the complete absence of staining was noted as negative. For AR and PSA, positive or negative staining was recorded. Three veterinary pathologists (CEFA, PEK and RLA) independently interpreted the immunohistochemical results.

Western Blotting

Frozen prostate samples were cut using a cryostat to confirm the presence of the tumor and then were mechanically homogenized for 30 s at 4°C in 50 mM Tris–HCl buffer pH 7.5, 0.25% Triton X-100 and EDTA using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Following centrifugation of the homogenate, protein was extracted from the supernatant and quantified as described by Bradford (1976). Equal amounts of protein (70 μg) from each sample were heated at 95°C for 5 min in sample loading buffer, subjected to SDS–PAGE or electrophoresis under reducing conditions and then transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO).

The blots were blocked with 3% bovine serum albumin in TBS-T (10 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 1 h and then probed overnight with p63 and HMWC antibodies. Goat anti-β-actin antibody (1:1,000; sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody, the blots were visualized via chemiluminescence (Amersham ECL Select Western Blotting Detection Reagent, GE Healthcare). Protein bands were quantified by densitometry and expressed as the integrated optical density (IOD). HMWC and p63 protein expression was normalized to β-actin expression. Normalized data are presented as the mean and standard deviation (SD).

Statistical analysis

The chi-square exact test was used to determine the significance of the differences in protein immunohistochemical expression between canine PC samples (p63+ and p63-) and normal samples.

Survival curves were generated using the Kaplan-Meier method, and statistical significance was determined using a log-rank test. Overall survival was defined as the period (in months) between the date of surgery and death caused by the disease. The T test was used to analyze the Western blotting data. $P<0.05$ was considered significant for all analyses. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) software was used for the statistical analysis.

RESULTS

Clinical Data

Clinical data were obtained from medical records (Supplementary Table 2). The mean age of dogs with p63+ PC was 11.7 years (8 to 15 years), and the mean survival time was 146.6 days (12 to 423 days) (Figure 1). Six animals (6/10) had metastatic disease at diagnosis. Of the four remaining animals, two underwent radical prostatectomy, and two received chemotherapy due to local invasion and the impossibility of surgical resection. In these cases, prostatic biopsies were performed for PC diagnosis. A few months later, these animals showed distant metastasis to the lungs and/or pelvic bones. Multiple metastatic sites were observed, including in the lungs (6/10), bones (5/10), intestine (2/10) and liver (1/10). At diagnosis, no significant difference was observed in the survival time between patients with and without metastasis.

The mean age of animals with p63- PC was 8.7 years (9 to 14 years), and the mean survival time was 328.6 days (74 to 523 days). Metastasis information was available for 70% (7/10) of these animals; four (4/7) had distant metastasis. One patient showed only bone metastasis, two showed bone and lung metastases, and one presented with lung and liver metastases. Three patients who were evaluated by X-ray and CT had no evidence of metastasis at diagnosis.

Histological Pattern

p63+ PC were classified as: cribriform pattern in 40% (4/10) of cases, small acinar pattern in 40% (4/10) of cases, and a mixed pattern (cribriform and small acinar) in 20% (2/10) of cases (Supplementary Table 2). Adjacent prostate tissue analysis revealed the presence of normal cells in 20% (2/10) of samples and PIA lesions in all cases. In contrast, PIN foci surrounding PC were not observed in

this set of samples. The Gleason-like score was 10 in 60% (6/10) of tumors, 8 in 20% (2/10) of tumors, and 6 in 20% (2/10) of tumors.

p63- PC showed a small acinar pattern in 40% (4/10) of cases, solid pattern in 30% (3/10) of cases, and cribriform in 30% (3/10) of cases. The Gleason-like score was 10 in 60% (6/10) and 6 in 40% (4/10) of the p63- tumors.

Expression of luminal and basal cell markers

Canine PC tissues (both p63+ and p63-) showed higher expression of p63 and HMWC compared to normal tissue samples. Western blotting analysis confirmed the overexpression of basal cell markers in p63+ canine PC (Figure 2).

Basal cell markers (p63, CK5 and HMWC), luminal cell markers (CK8/18, AR and PSA) and a proliferative marker (Ki67) were investigated in all the prostate samples (Table 1). All the normal samples contained p63-positive cells in the basal layer. Nevertheless, these cells were discontinuous (Figure 2e). The same pattern was observed for CK5 and HMWC, with a discontinuous basal cell layer in normal prostatic tissue (Figure 2f). The luminal epithelial cells were negative for all basal markers (Table 1, Figure 2e and f). High CK8/18 expression was observed in luminal cells, while basal cells were negative for this marker. The epithelial and basal cells from normal tissues showed low Ki67 score (score 2 or 3) (Table 1).

Immunohistochemical results for p63+ PC are shown in Table 2. Cancer cells showed higher expression of p63, CK5 and HMWC, than normal tissue and p63- PC. Interestingly, continuous staining of basal cells was observed in tumor samples, unlike what was found in normal tissue (Figure 2h; Figure 3a and Supplementary Figure 1). In 100% (10/10) of the samples, strong membranous and cytoplasmic HMWC expression was observed (Figure 2h and Figure 3c); 40% (4/10) showed aberrant cytoplasmic p63 expression, and 30% (3/10) received a score 3 for CK5 cytoplasmic expression. Four tumor samples (40%) were negative for AR, and 60% were positive (Figure 4). Among the PC tissues, 50% (5/10) were negative for PSA, and the other 50% (5/10) contained some PSA-positive cells (Table 1). All the prostatic tumors with aberrant p63 expression showed positive co-expression of CK8/18 and basal markers.

The immunohistochemical evaluation of p63- canine PC samples is summarized in Table 1. All the tumor cells were negative for p63 (10/10). Two tumor tissues contained CK5-positive cells (score 1),

and all the tumor samples (10/10) showed cytoplasmic expression of HMWC and moderate (score 2) to strong (score 4) CK8/18 expression.

Protein Expression Analysis

Luminal cells in normal prostatic tissues showed strong nuclear PTEN staining (score 3 or 4), strong NKX3.1 staining (score 3 or 4) and low (score 1) to negative cytoplasmic phospho-AKT staining. Normal luminal cells were negative for Chromogranin, and CD44+/CD24- cells were absent. Normal prostate tissue also showed low cytoplasmic C-MYC staining. In normal prostate tissues, basal cells were negative for PSA, but all the luminal cells were strongly positive for PSA and AR. The immunohistochemical evaluation of the normal samples is summarized in table 1.

The immunohistochemical results for the non-basal markers are summarized in Table 2 and Figure 4. p63+ PC samples showed loss of nuclear PTEN staining (Figure 4) compared to normal tissue samples (P=0.0001). Negative to weak cytoplasmic PTEN positive staining was observed in canine PC. All PC tissues showed strong cytoplasmic phospho-AKT (Ser473) expression (P=0.002) (Figure 4) and nuclear C-MYC staining (P=0.001). All p63+ PC samples were negative for NKX3.1 (P=0.0001) (Figure 4) and Chromogranin and contained neoplastic cells with the CD44+/CD24- phenotype.

Fifty percent of p63- PC samples (5/10) were positive for PTEN, and cytoplasmic phospho-AKT (Ser473) expression was moderate (60%; 6/10) or high (40%; 4/10). Thirty percent of these tumor samples (3/10) were positive for NKX3.1. The tumor samples showed strong (score 3) C-MYC expression, and four samples (4/10) contained CD44+/CD24- cells (Table 2). The protein expression comparison between human and canine PC aberrant p63 expression is shown in Supplementary Table 3.

DISCUSSION

Canine PC has been considered a model for human prostatic diseases. In contrast to laboratory animals, dogs spontaneously develop tumors and provide a unique opportunity for comparative studies. Recently, Akter et al. (2015) described the basal cell marker CK5 in canine prostate carcinoma, although most tumor samples contained CK8/18-positive cells, suggesting a luminal cell origin. In our study, 13.7% of all tumors that we have in our archive was p63+ (10/74). In canine PC, there are no data on the

use of p63 as a diagnostic or prognostic marker. Herein, we confirmed p63 and HMWC expression in neoplastic prostatic cells by Western blotting. We also reported negative PSA expression in 50% (5/10) of p63+ PC samples, indicating a non-secretory phenotype, although all of the tumors were positive for both luminal (CK8/18) and basal (CK5) cell markers.

In addition to the nuclear expression of p63 by neoplastic cells, three tumors (3/10) showed aberrant cytoplasmic p63 expression. These patients experienced a shorter survival (49 days) compared to the whole group (146.6 days), and two of these dogs developed metastatic disease. Romanucci et al. (2014) found that 27.2% (3/11) of canine PC tumors had cytoplasmic p63 expression, which was associated with Hsp60, Hsp72 and Hsp7 expression. The authors concluded that basal cells play an important role in the development of canine PC. In human PC, cytoplasmic p63 expression was correlated with increased mortality (Dhillon et al. 2009).

The prostate epithelium contains luminal, basal and rare neuroendocrine cells, and prostate cancer may originate from one of these cell types. Here, the neuroendocrine origin of these tumors was refuted since there was no Chromogranin staining in neoplastic samples (both p63-positive and p63-negative tumors).

We demonstrated diffuse HMWC expression in both p63+ and p63- PC. In p63+ PC, CK5 was diffusely positive in three cases (3/10), but there were fewer than 30% positive tumor cells in seven of the cases. In p63- PC specifically, only two (20%) cases were positive for CK5. Akter et al. (2015) showed CK5-positivity in 13 of 20 canine PC samples, which represent a higher frequency than that reported herein. However, these authors did not perform p63 staining of tumor samples and thus could not evaluate these tumors in terms of p63 expression pattern. This previous study reported that 25% of the PC samples were positive for CK5 and that 20% were positive for CK14. According to the authors, the positive expression of these basal cell proteins is consistent in different histological PC types, and the role of these cells should be considered in canine carcinogenesis. In humans, Tan et al. (2015) reported no expression of HMWC or CK5 and luminal expression of CK18 in human p63+ PC. In overall, these data suggest that canine PC has a different immunoexpression pattern than tumors of same type in humans, which are negative for HMWC and CK5 and positive for CK8/18. It is possible that p63+ canine PC is derived from luminal cells (CK8/18) that are dedifferentiating and thus gaining basal cell marker expression (HMWC and CK5). Conversely, these tumors could arise from basal cells that are differentiating into luminal cells.

We also evaluated p63, CK5 and HMWC immunostaining in canine metastases from these animals (data not shown). All the metastases were negative for CK5 and positive for HMWC and p63 (Supplementary Figure 2). Akter et al. (2015) reported one canine PC sample (CK14, CK8/18, CK5 and AR) with neoplastic emboli showing diffuse expression of CK5. The authors suggested that CK5-positive cells play a role in canine metastasis. Among our p63-negative PC cases, only two were positive for CK5; one of these animals presented with metastatic disease (case 14), and there was no information available for the other (case 16). The other three animals in this group had metastatic disease and presented CK5-negative tumor cells.

In our study, both p63+ and p63- tumors presented similar results in terms of AR immunostaining (10 negative/low cases and 7 negative cases, respectively); suggesting that canine PC is hormone independent. In humans, AR-negative PC is refractory to hormone therapy; it has been associated with a more aggressive outcome (Hu et al. 2009) and could be associated with cancer stem cells (CSCs). To evaluate the presence of CSCs in our cases, we performed CD44 and CD24 immunostaining; all PC samples had CD44+/CD24- phenotype, supporting the CSC hypothesis. Schroeder et al. (Moulay et al. 2013) showed a correlation between androgen receptor loss and the stem cell-like phenotype in human PC. Moulay et al. (2013) reported that CD44 is a promising CSC marker in canine prostate tumors. Our group of tumors (both p63+ and p63-) showed no correlation between loss of AR expression and gain of the CSC phenotype. Normal prostatic tissue contains more AR-positive cells and no CD44+/CD24- cells. Interestingly, all p63+ PC cases exhibited the CSC phenotype, compared with only 40% of p63- PC cases.

PTEN loss has been associated with phosphatidylinositol-3,4,5-triphosphate (PIP3) and AKT activation (Dubrovska et al. 2009), which is related to apoptosis, cellular proliferation and tumorigenesis (Majumder et al. 2005). Lu et al. (2013) demonstrated that conditional ablation of PTEN promotes basal-to-luminal differentiation and invasive PC in a mouse model. Our p63+ canine PC cases showed loss of nuclear PTEN staining and an accumulation of cytoplasmic AKT, suggesting that these tumors could have continuous activation of the PI3K/AKT pathway. In addition, PTEN-negative tumor cells showed a basal cell phenotype (p63+/CK5+/HMWC+) and do not differentiate into luminal protein-expressing cells. *PTEN* genomic loss, which leads to constitutive activation of the PI3K/AKT pathway, and 8q amplification, which includes the *MYC* gene, occur in approximately 30% of human prostate tumors,

representing the most frequent genetic alterations in prostate cancer (Priolo et al. 2014). *MYC* and *AKT* are arguably the most prevalent driver oncogenes in prostate cancer, and our results suggest that MYC overexpression occurs in addition to PTEN loss and AKT activation, thereby conferring a more aggressive phenotype to this group of tumors.

Human p63+ PC showed positive NKX3.1 immunostaining, which differs from the usual type of PC (Tan et al. 2015). We previously reported negative NKX3.1 immunostaining in canine prostatic lesions (Fonseca-Alves et al. 2013). Herein, NKX3.1 negativity was not associated with aberrant p63 expression; one plausible explanation involves the cell of origin because normal basal cells are negative for NKX3.1 (Priolo et al. 2014).

CONCLUSIONS

A specific subset of canine prostatic carcinomas, p63+ is reported herein. These tumors are less frequent in dogs than in humans, but canine prostatic carcinomas that aberrantly express p63 have a different phenotype, such as positive expression of CK5 and high molecular weight cytokeratin as well as negative expression of PSA, NKX3.1 and AR. This information contributes to a better understanding of the cellular origin of prostatic carcinoma and to a better characterization of this specific type of prostatic carcinoma in dogs.

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Table 1. Immunohistochemical characterization of luminal and basal markers in normal canine prostate tissues and neoplastic prostate cells according to p63 expression.

	Basal markers			**Luminal markers**			**Proliferative marker**
Identification	**P63**	**CK5**	**HMW**	**CK8/18**	**AR**	**PSA**	**Ki67**
P63-positive PC							
1	3	4	4	3	0	1	3
2	3	2	4	4	1	0	3
3	2	2	4	3	1	1	2
4	2	4	4	4	1	0	3
5	2	2	4	2	0	0	2
6	2	2	4	3	1	0	3
7	3	2	4	3	1	1	2
8	2	2	4	2	0	0	3
9	2	4	4	3	1	1	3
10	3	3	4	3	0	1	2
P63-negative PC							
11	0	0	2	3	0	1	2
12	0	0	3	4	2	1	3
13	0	0	4	3	0	1	4
14	0	1	2	4	0	1	3
15	0	0	4	3	0	1	4
16	0	1	3	4	0	1	3
17	0	0	4	3	0	1	4
18	0	0	2	4	1	1	3
19	0	0	3	3	1	1	4
20	0	0	2	2	0	1	3
Normal prostate							
1	0	0	0	4	4	1	0
2	0	0	0	4	4	1	0
3	0	0	0	4	4	1	0
4	0	0	0	4	4	1	0
5	0	0	0	4	4	1	0
6	0	0	0	4	4	1	0
7	0	0	0	4	4	1	0
8	0	0	0	4	4	1	0
9	0	0	0	4	4	1	0
10	0	0	0	4	4	1	0

Table 2. Immunohistochemical results of prognostic markers in normal canine prostate tissue and in human prostate cancer according to p63 expression.

Identification	**PTEN**	**AKT**	**C-MYC**	**NKX3.1**	**CD44+/CD24-**	**Chromogranin**
P63-positive PC						
1	0	4	4	0	2	0
2	1	4	4	0	1	0
3	1	4	4	0	2	0
4	0	4	4	0	1	0
5	0	4	4	0	1	0
6	1	4	4	0	2	0
7	0	4	4	0	1	0
8	1	4	4	0	2	0
9	0	4	4	0	2	0
10	0	4	4	0	1	0
P63-negative PC						
11	0	3	4	1	0	0
12	2	3	4	0	2	0
13	0	4	4	0	0	0
14	1	3	4	0	0	0
15	0	4	4	0	2	0
16	1	3	4	1	0	0
17	0	4	4	0	1	0
18	2	3	4	0	0	0
19	0	4	4	0	1	0
20	1	3	4	1	0	0
Normal prostate						
1	3	1	1	4	0	
2	4	1	1	4	0	0
3	3	0	1	4	0	0
4	4	1	1	4	0	0
5	3	0	1	4	0	0
6	4	1	1	4	0	0
7	3	0	2	4	0	0
8	4	1	1	4	0	0
9	3	0	2	4	0	0
10	4	1	1	4	0	0

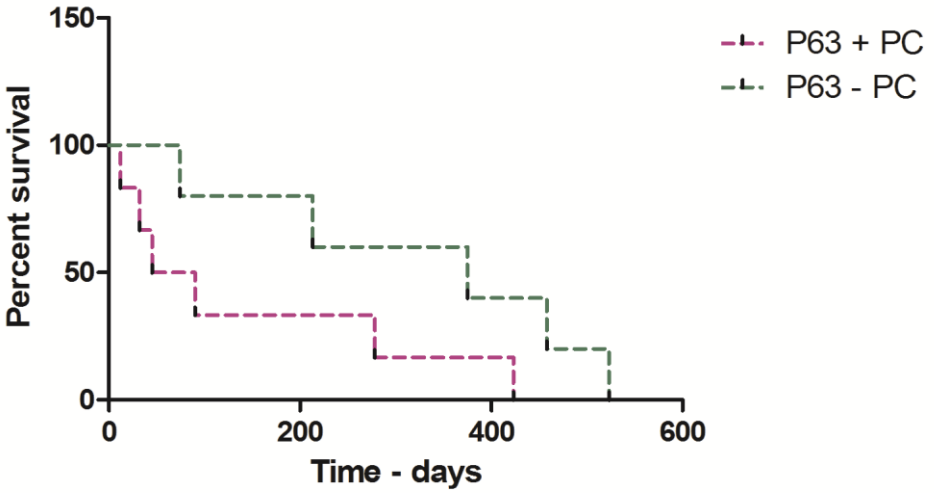


Figure 1. Kaplan-Myer analysis of patients with prostate cancer (PC) showing aberrant p63 expression versus patients with PC negative to p63 staining. It is possible to observe two separate curves and PC patients showing aberrant p63 expression experienced a lower survival time.

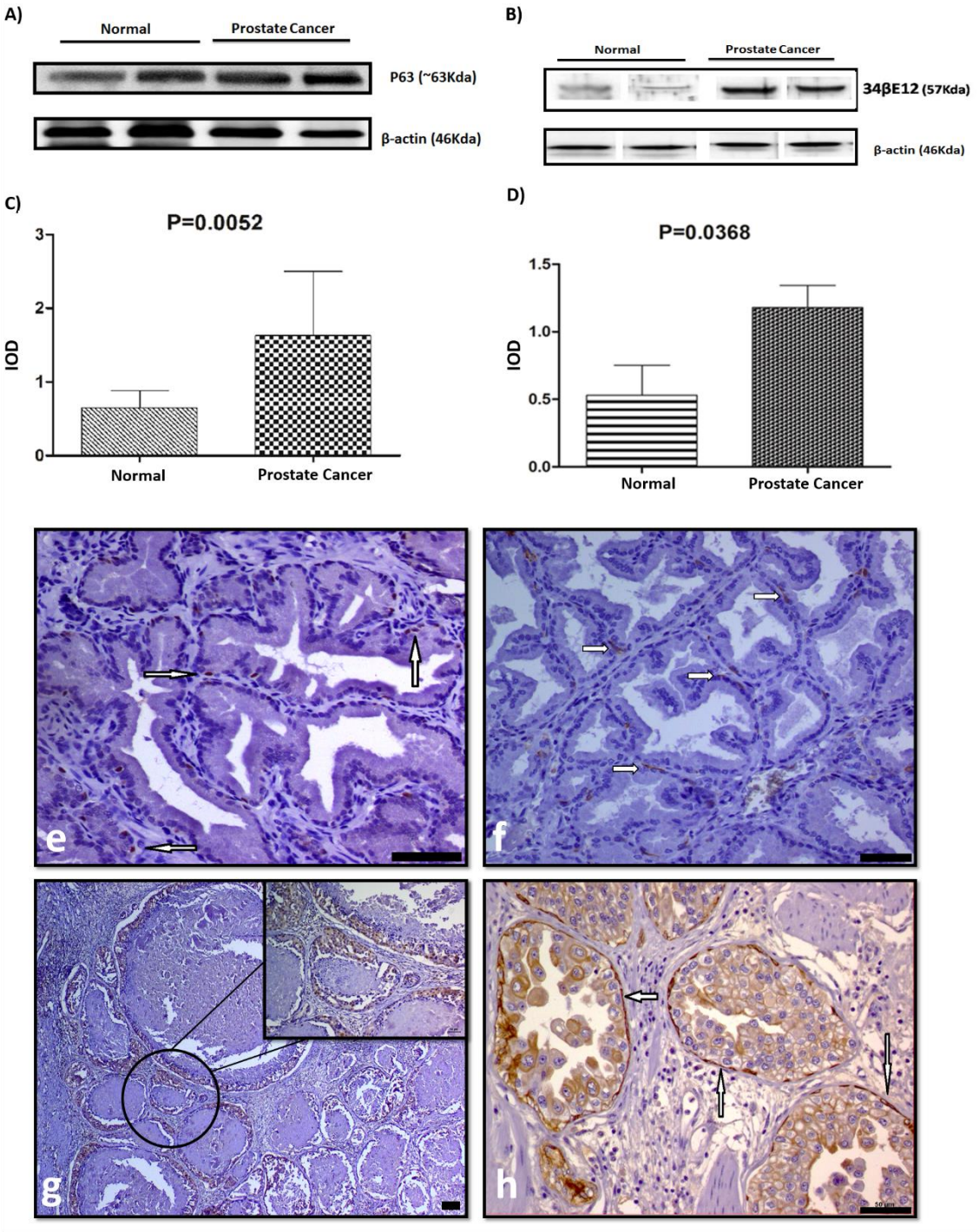


Figure 2. Characterization of basal cells markers in normal prostate samples and canine p63+ PC. The Western blotting analysis revealed higher p63 expression in tumor samples compared to normal samples (A and C) and higher expression of HMWC in tumor samples compared with normal samples (B and D). Normal prostate. The immunohistochemical analysis showed a discontinuous basal cells layer (arrows) that was positive for p63 (e) and HMWC (f). Canine p63+ PC cells showed aberrant expression of p63 (g) and HMWC (h). The continuous basal cells layer in the tumor samples are indicated by arrows (h).

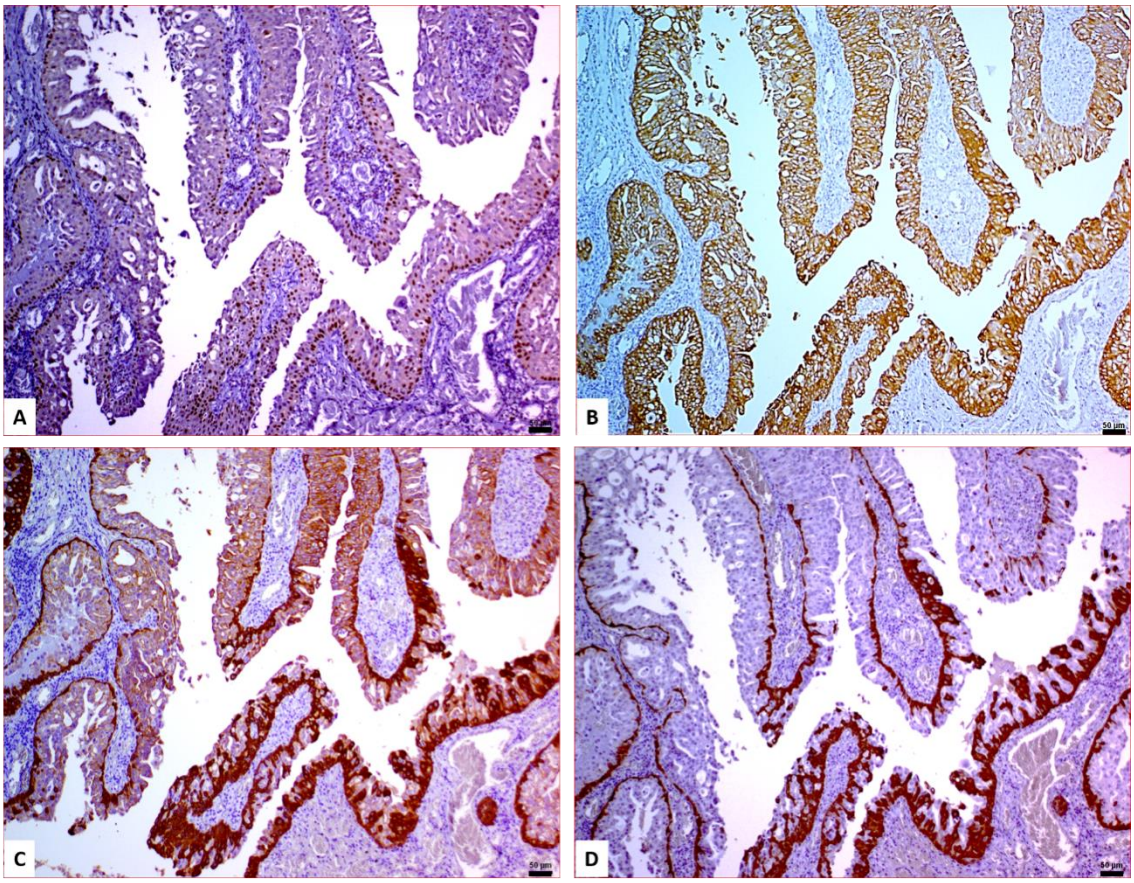


Figure 3. Immunohistochemical staining for p63 (A), CK8/18 (B), HMWC (C) and CK5 (D) in canine p63+ prostate cancer. Strong positive p63 staining was observed in a continuous basal cells layer (A). The subsequent section demonstrates continuous positive CK8/18 staining in the same cells (B). Neoplastic tissues showed strong expression of HMWC (C) and CK5. Peroxidase, DAB, Harry's hematoxylin Counterstain, 10X.

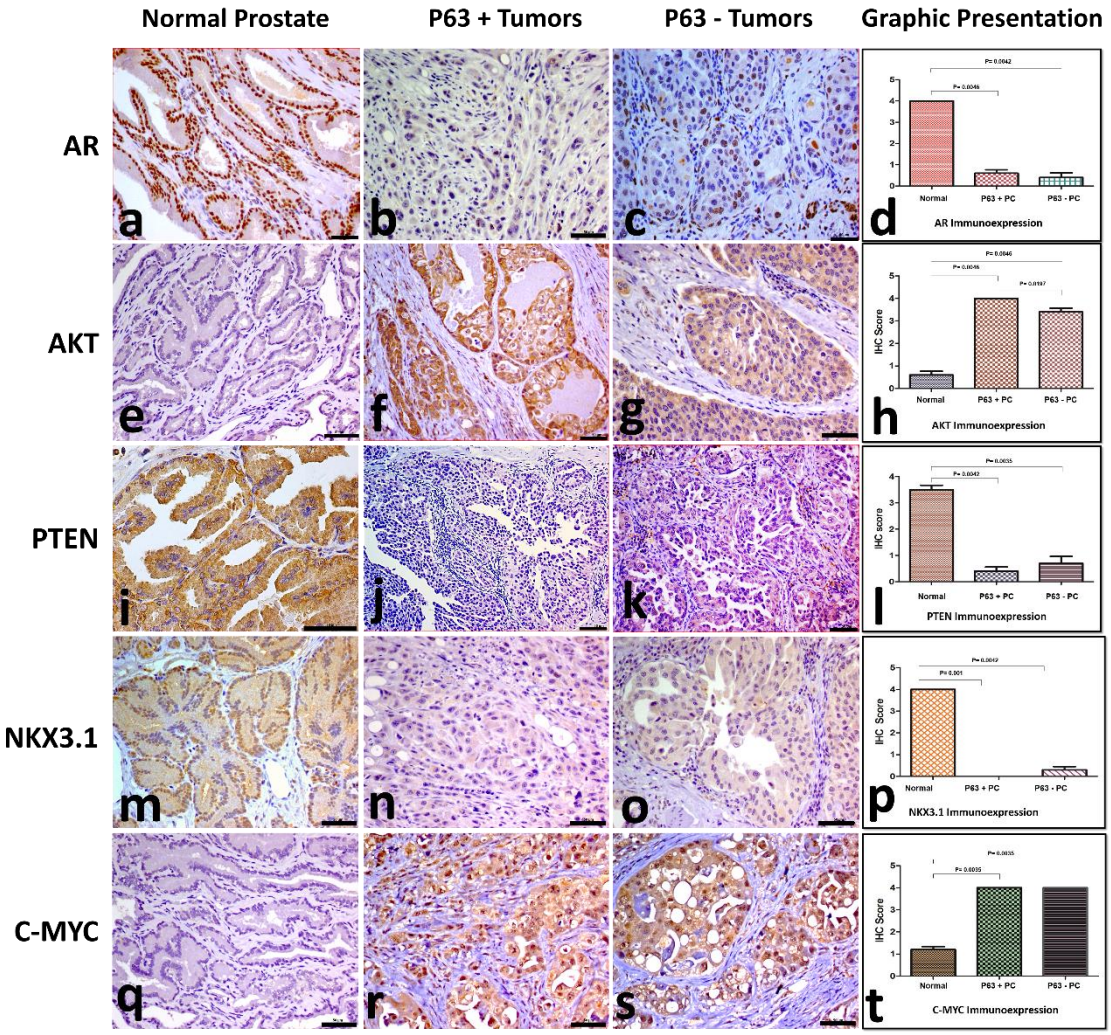


Figure 4. Immunohistochemical staining for AR, AKT, PTEN, NKX3.1, and C-MYC in canine prostate cancer. It is possible to observe a strong expression of AR in normal prostatic tissue (a and d) and rare neoplastic and basal cells were positive for AR in p63+ PC (b and d) and p63- PC (c and d). Normal prostatic tissue showed AKT negative (e) to low expression (h) protein and it was possible to note a strong expression in p63+ PC (f and h) and tumor negatives against p63 protein (g and h). Normal prostatic tissue presented strong expression of PTEN (i and l) and NKX3.1. p63+ PC (j and l) and PC (k and l) p63-negative showed low PTEN expression. All p63+ PC was negative for NKX3.1 staining (n and p) and p63-negative PC showed negative NKX3.1 expression (o and p). Normal prostatic tissue showed negative (q) to low (t) C-MYC expression and it was possible to note a higher expression in both p63+ (r and t) and p63- (s and t) tumors.

Supplementary Table 1. Primary antibodies used for immunohistochemical analysis.

Antibody	Clone	Dilution	Manufacturer
P63	4A4	1:150	Dako Cytomation
CK5	XM26	1:50	Thermo Scientific
HMWC*	34βE12	1:100	Dako Cytomation
CK8/18	5D3	1:600	Novocastra
Ki67	MIB-1	1:50	Dako Cytomation
Chromogranin	Polyclonal	1:500	Abcam
CD44	IM7	1:50	Santa Cruz
CD24	M1/69	1:75	Santa Cruz
PTEN	MMAC1	1:500	Bioss
AKT	Ser473	1:50	Cell Signaling
C-MYC	C-19	1:100	Santa Cruz
NKX3.1	P050	1:50	Aviva
PSA	Polyclonal	1:800	Bioss
AR	ab77557	1:100	Abcam

*High molecular weight cytokeratin

Supplementary Table 2: Clinical and histological data of prostate cancer from 20 dogs.

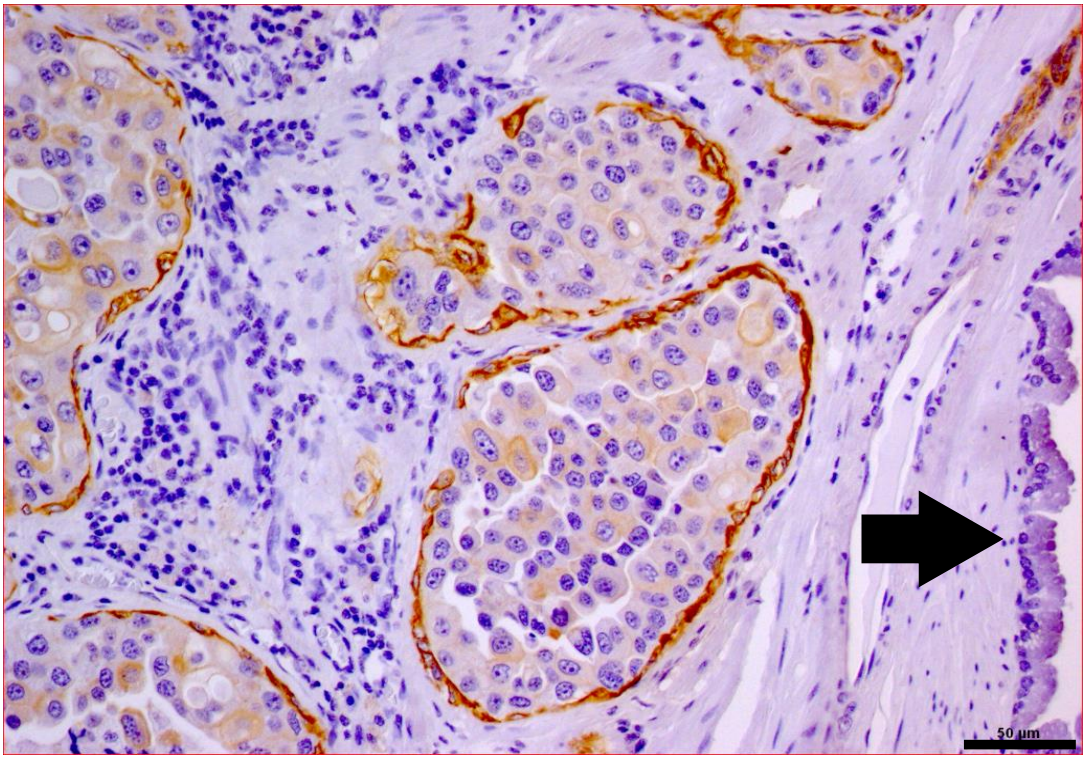
Case	Breed	Age	Metastasis	Histological Pattern	Gleason-like score	Treatment	Outcome (days)
PC with aberrant p63							
1	Boxer	14	Lung, Bone and Liver	Cribiform	10	Piroxicam	90
2	Teckel	11	No	Cribiform	10	N/A	N/A
3	Boxer	12	Bone, Lung	Cribiform	10	LDMT	278
4	MBD	15	Bone, Lung	Cribiform	10	LDMT	423
5	MBD	13	Bones, Intestine, Lung	Small acinar + cribiform	8	N/T	12
6	German Shepherd	10	No	Small acinar + cribiform	8	N/T	N/A
7	Poodle	8	No	Small acinar	6	RP	45
8	American Cocker Spaniel	10	No	Small acinar	10	RP	32
9	American Pitbull Terrier	10	Bones, Lung	Small acinar	6	Piroxicam	N/A
10	MBD	14	Lung, intestine	Small acinar	10	N/A	N/A
PC with negative-p63							
11	MBD	9	No	Small acinar	6	LDMT	523
12	Boxer	11	Bone	Small acinar	6	Piroxicam	N/A
13	Poodle	10	No	Solid	10	RP	213
14	Boxer	13	Bone, lung	Small acinar	6	N/T	74
15	German Shepherd	12	Bone, lung	Solid	10	LDMT	375
16	MBD	14	N/A	Small acinar	6	N/A	N/A
17	MBD	12	N/A	Cribform	10	N/T	N/A
18	MBD	10	Lung, liver	Solid	10	Piroxicam	458
19	American Pitbull Terrier	9	N/A	Cribform	10	N/T	N/A
20	MBD	13	No	Cribform	10	N/A	N/A

PC prostate cancer; MBD Mixed Breed dog; N/A Not Available; N/T No Treatment; RP Radical Prostatectomy; LDMT Low-dose metronomic therapy

Supplementary Table 3. Comparison between previous results of aberrant p63 expression in human and canine PC.

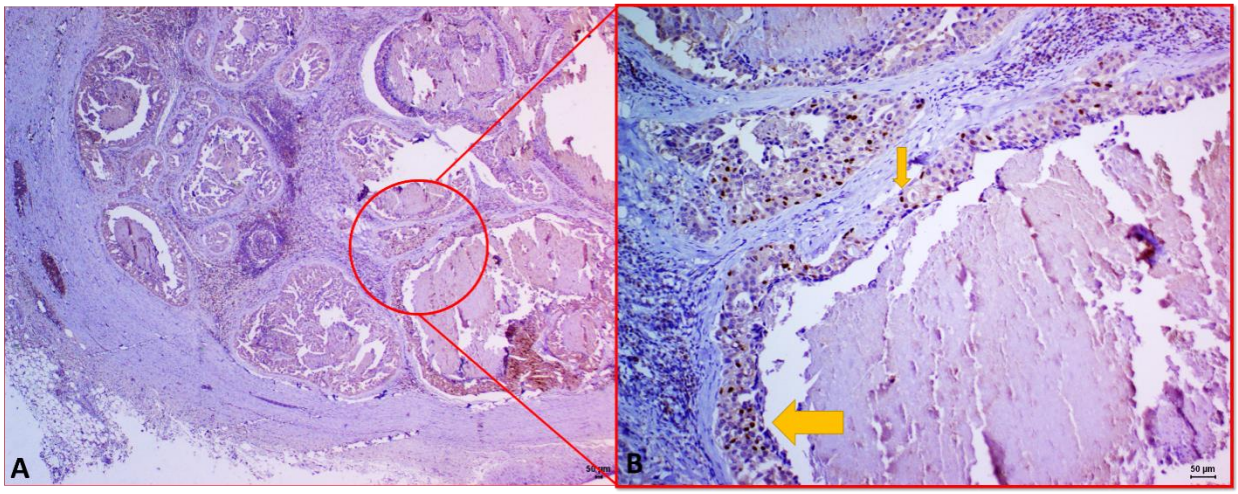
Human PC: p63+	**Dog: normal prostate**	**Dog PC: p63+**
p63 - positive[*,¥,∞]	p63 – positive; discontinuous	p63 - positive
HMWC - negative[*,¥,∞]	HMWC - positive; discontinuous	HMWC - positive; continuous
CK5 – low to negative[*]	CK5 – positive; discontinuous	CK5 – positive; continuous
AR - positive [*]	AR – positive	AR – negative to positive
NKX3.1 - positive[*]	NKX3.1 – positive	NKX3.1 - negative
PSA - positive[¥,∞]	PSA – positive	PSA - negative
PTEN - negative[*]	PTEN – positive	PTEN – negative to low
AKT – no data	AKT – low to negative	AKT – strongly positive
cMYC – no data	cMYC - absent or low	cMYC – strongly positive
CD44+/CD24- no data	CD44+/CD24- absent	CD44+/CD24- present
KI67 - low[¥,∞]	Ki67 – low	Ki67 - High

PC: prostate carcinoma. *Tan et al. (2014); ¥Wu and Kunju (2012); ∞Osunkaya et al. (2008).



Supplementary Figure 1. Canine prostate cancer with solid pattern showing cytoplasmic expression of high molecular weight cytokeratin (HMWC) and a continuous basal cells layer showing remarkable membranous staining for HMWC. It is possible to observe adjacent normal prostatic tissue showing no expression of HMWC in the epithelial cell cytoplasm and basal cell layer (black arrow).

Supplementary Figure 2. p63 immunostaining in a lymph node metastasis of canine prostate cancer with



aberrant p63 expression. It is possible to observe p63-positive epithelial cells invading the lymph node parenchyma (A). B: p63 nuclear immunostaining in neoplastic cells in the same section at higher magnification (20x).

CAPÍTULO IV

ARTIGO CIENTÍFICO 3

TRABALHO CIENTÍFICO A SER SUBMETIDO PARA A REVISTA "AMERICAN JOURNAL OF PATHOLOGY"

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E-cadherin transcriptional downregulation in canine prostate cancer occurs in malignant progression due to CDH1 methylation

Abstract

Canine prostate cancer (PC) is an aggressive disease with high metastatic rate and poor prognosis for the patients. E-cadherin is a transmembrane glycoprotein responsible for cell-to-cell adhesion and its loss is associated with metastatic process. We evaluate E-cadherin gene and protein expression in canine PC compared to normal tissue and evaluate DNA hypermethylation as a regulatory mechanism of *CDH1*. We found low E-cadherin protein expression by WB and low *CDH1* transcript levels in PC samples, compared to normal prostatic tissue. We also performed immunohistochemistry and found membranous E-cadherin in normal and PC samples. We did not find statistical difference between groups. Thus, we used a percentage of E-cadherin negative cells and we identified a relation between the number of negative cells, Gleason score and survival time. We found *CDH1*hypermethylation in canine PC when compared with normal samples. Our results suggests a dynamic expression of E-cadherin in PC with *CDH1*hypermethylation responsible for E-cadherin silencing.

Key-words: Dog, *CDH1,* prostatic disease, metastasis.

Introduction

Cell-cell adhesion is essential in epithelial cells development, differentiation and survival[1]. The carcinogenic process, from normal to pre neoplastic and invasive carcinoma, involves the ability of epithelial cells to detach one another, survive and invade the surrounding tissue[2].E-cadherin is a transmembrane protein that has a key role in cell adhesion and migration[3]. E-cadherin also is involved in different pathways, such as β catenin/APC, related to cell proliferation and epithelial mesenchymal transition (EMT)[4]. Loss of E-cadherin is associated with poor prognosis in patients with PC and high-grade tumors[5,6]. DNA hypermethylation is implied with *CDH1*silencing and E-cadherin down regulation[3].

Besides the development of PIA lesions as a preneoplastic lesion, human and dogs shares many similarities in PC development[7]. Human PC has a changeable behavior and is second leading cause of deaths related with male cancers in North America[8]. This mortality rate is associated with metastasis[9]. The most common metastatic sites in humans are bones, lymph nodes and lungs[8]. Metastasis of prostate cancer is a complex process associated with loss of epithelial markers, acquirement of a mesenchymal phenotype and the ability of cells to spread through the lymphatic system or the bloodstream[10]. Canine prostate cancer it is a very aggressive disease associated with high metastatic rate at the diagnosis (more than 85%) and bones and iliac lymph node are the most common metastatic site[7.]

.Loss of E-cadherin is one of the most important events to metastasis development[7]. Our research group has investigated the role of E-cadherin in

canine prostate cancer[7,11-13]. We demonstrated loss of E-cadherin during lymphatic invasion by neoplastic epithelial cells and E-cadherin re-expression in metastatic foci, indicating a dynamic E-cadherin expression in metastatic process[7].

Different mechanisms have been implicated with E-cadherin downregulation in human medicine, however, there are few studies evaluating the molecular mechanisms involved in E-cadherin silencing in dogs. Methylation of *CDH1* promoter is widely studied as a cause of E-cadherin down-regulation in human PC[14,15]. Methylation is an epigenetic mechanism associated with the incorporation of a methyl group in specific promoter gene regions[16]. This promoter region is a CpG islands and shows a key role to epigenetic instability[17]. The hypermethylation in the promoter region of a suppressor tumor gene is responsible for gene silencing and lack of protein expression. However, this phenomenon is reversible, and the neoplastic cells can induce hypomethylation and re-express the respective proteins[18]. Our research aimed to evaluate E-cadherin gene and protein expression in canine PIA, PC and its metastasis as well the methylation status of *CDH1* as a silencing mechanism responsible for the dynamic E-cadherin expression.

Material and Methods

Tissue selection and Histopathological evaluation

We selected from our Veterinary Pathology archive (Univ. Estadual Paulista-UNESP) 67 normal samples, 67 PIA samples, 67 PC samples and five metastatic foci. We collected the PC samples and metastasis during prostatectomy surgery or biopsies procedure from animals showing clinical

signs and complementary exams indicating a neoplastic process. Normal and PIA samples were collected during necropsy from animals without clinical signs of prostatic disease. All prostate samples were from intact dogs.

The histopathological classification was performed according to human WHO classification of Tumors of the Urinary System and Male Genital Organs[19] and the Gleason-like system was applied according to Palmieri and Grieco[20]. The animals procedures were approved by the Animal Ethics Committee of the University of Sao Paulo State (UNESP).

Immunohistochemistry

We performed E-cadherin immunohistochemical analysis in 20 normal prostates, 20 PIA lesions, 20 PC and five metastasis. Slide sections were dewaxed in xylene and rehydrated in graded ethanol. For antigen retrieval, the slides were incubated with citrate buffer (pH 6.0) in a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA). The slides were treated with freshly prepared 3% hydrogen peroxide in methanol for 20 min and further washed in Tris-buffered saline. The E-cadherin antibody (Monoclonal Mouse Anti-Human E-Cadherin, Clone NCH-38) was diluted in 1:300 and was incubated over night at 4°C. A polymer system (Envision, Dako, Carpinteria, CA, USA) was applied as a secondary antibody conjugated to peroxidase and 3´-diaminobenzidine tetrahydrochloride (DAB1, Dako, Carpinteria, CA, USA) was used as the chromogen, for 5 min, followed by Harris hematoxylin counterstain. Negative controls were performed omitting the primary antibody and replacing them with Tris-buffered saline. Normal prostate was used as positive control.

Immunohistochemical Scoring

The slides were analyzed under a light microscope (Leica Microsystems, Germany) and five images were taken for each slide through Leica QWin V3 software (Leica Microsystems, Germany) at high-power (40X objective) field. Representative areas were qualitatively selected for immunostaining analysis. We choose areas with minimal inflammatory cells, necrosis or connective tissue and with lower E-cadherin staining. These images were analyzed by ImageJ computational program. Staining was evaluated by establishing a "threshold" using the ImageJ threshold tool. Furthermore, we counted a number of epithelial negative cells for E-cadherin antibody in normal, PIA, PC and metastasis. The samples were scored to immunohistochemistry based on an assessment of the number of negative cells per the total of cells in five high power field (HPF). These results were expressed in a percentage of negative cells.

Gene expression

We performed *CDH1* gene expression using 20 normal prostate, 20 PIA lesion and 20 PC and five metastasis from formalin-fixed and paraffin-embedded (FFPE) tissue. All FFPE samples were macrodissected using 16 gauge needles and mRNA were extracted using a commercial RecoverAll™ Total Nucleic Acid Kit (Ambion, Life Technologies, MA, USA) according to the manufacturer's instructions. The mRNA concentration was determined with a spectrophotometer (NanoDrop™, ND-8000, Thermo Scientific, MA, USA), while the mRNA 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 50ºC, and the enzyme was subsequently inactivated for 15 min at 70ºC. cDNA was stored at -80ºC.

CDH1 (Forward: 5'-CAGCATGGACTCAGAAGACAGAAG-3' and Reverse: 5'-TTCCGGGCAGCTGATAGG-3') and the endogenous (*ACTB*, Forward: 5'-GGCATCCTGACCCTCAAGTA-3' and Reverse: 5'-CTTCTCCATGTCGTCCCAGT-3') genes were 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. Relative gene expression was quantified using the 2-ΔΔCT method[21].

Western blotting

We performed Western blotting in seven normal prostate, seven PIA lesions and seven PC to evaluate the E-cadherin protein expression. The frozen prostate samples were cut in a cryostat to confirm the previous diagnosis and then were mechanically homogenized to nitrocellulose membranes as a

previous described (Rivera-Calderon et al., 2016).The blots were blocked with 6% skimmed milk in TBS-T for two hours and E-cadherin antibody was incubated for 18 hours. Goat anti-β-actin antibody (1:1:000; sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a positive 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). Protein bands were quantified by densitometry analysis and expressed as integrated optical density (IOD). KIT protein expression was normalized to the β-actin values. Normalized data are expressed as the means and standard deviation (SD).

Quantitative bisulfite pyrosequencing

The pyrosequencing analysis was performed in E-cadherin gene (*CDH1*) to evaluate the frequency of methylation in their promoter. We used 20 normal prostates, 20 PIA samples and 20 PC. The frozen prostate samples were cut in a cryostat to confirm the previous diagnosis. We performed the bisulfite conversion of the genomic DNA using EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Irvine, CA, USA). We perfumed a forward primer (5' TTTGGGAAGAGGAGGGGG 3') and reverse primer (5' CCCTTCCCCTCTCTCTCTC - BIOTIN 3') from *CDH1*CpGisland and amplified using PCR (HotStarTaq Master Mix kit - Qiagen). The pyrosequencing was performed using a sequencing primer (5' TTTGGGAAGAGGAGGGGG 3') following the manufacturer's instructions (PyroMark ID Q96, Qiagen and Biotage, Uppsala, Sweden).

Tumor-derived cell cultures

Cell cultures were established using fresh tissue from two PC from a 10-years-old, intact, mixed breed dog showing a non-metastatic PC and an 11-years-old, intact, poodle dog showing a metastatic PC. These tumors samples were evaluated by pyrosequencing previously and we confirmed hypermethylation of *CDH1*. The fresh tissues were washed three times with Dulbecco's Phosphate Buffered Saline Modified with 1% of 100 U/mL penicillin G and 100 mg/mL streptomycin (SIGMA, Portland, OR, USA). Fragments (2 mm) were dissociated using 0.5 % of collagen type IV (Sigma, St. Louis, MO, USA) for three hours and incubated at 37 °C in 5 % CO2.Cells were suspended in culture medium with 100 U/mL penicillin G and 100 mg/mL streptomycin (SIGMA, Portland, OR, USA). Cells were incubated at 37° C in 5% CO2 in culture medium supplemented with 10% of inactivated fetal bovine serum (FBS, HYCLONE, Waltham, MA, USA) and 100 U/mL penicillin G and 100 mg/mL streptomycin (SIGMA, Portland, OR, USA).The culture medium was discarded and replaced by fresh medium every 48 h.

The cell cultures immunophenotype was evaluated in the respective primary tumors using pancytokeratin (AE1/AE3, Invitrogen) at 1:300 dilution, P63 (Dakocytomation) at 1:100 dilution, PSA (Dakocytomation) at 1:2000 dilution and vimentin (Dakocytomation) at 1:300 dilution. We suspended the cell culture and performed a cellblock using agarose 1%. We performed 4μ paraffin section and performed the immunohistochemistry using citrato buffer pH 6.0 in a pressure cocker (Pascal® - Dakocytomation). Envision system (Dakocytomation) was used as secondary antibody. We used a normal prostate

as a positive control for all antibodies. Negative control was performed replacing the primary antibody with non-reactive antibody.

Methyl thiazolyl tetrazolium (MTT) assay

The MTT assay was performed to evaluate the 5-Aza 2′deoxycytidine (5-Aza-dC) effects on cell viability. We used a 96-well plates to grow the cancer cells at a density of 2,500 cells per well. The medium were changed each 48 hours and 5-Aza-dC it was added every 24 hours. MTT analysis was performed at day 7. The medium was removed and cells were washed with 3X PBS and fresh medium was added in each well and incubated at 37°C for 4 h. The medium was removed and 200µl of dimethyl sulfoxide (DMSO) was added in each well and formazan was solubilized. The optical density (OD) level was measured at 570. Each treatment was performed in triplicates and the experiment repeated a twice. Cell viability was calculated into percentage.

5-Aza-2′-deoxycytidine treatment

To investigate if hypermethylation could be a*CDH1* silencing mechanism, we treated our cell cultures with DNA demethylating nucleoside analog 5-Aza-dC. 1µg was added to the culture medium as previous established by MTT assay. We added 1µg every 24 hours due to 5-Aza-dC stability. After seven days of treatment, cells were washed with PBS three times, we performed trypsinization and cells were suspended in PBS for DNA extraction and methylation analyzes was done.

Data analysis

Statistical analysis was performed using GraphPad Prism v.5.0 (GraphPad Software Inc., La Jolla, CA, USA). Kruskal-Wallis or Mann-Whitney tests was applied to compare E-cadherin transcript levels among normal, PIA and PC samples. T test was used to determine the significance of the association between each E-Cadherin expression and the prostatic tissues. We performed correlation among the IHC score and clinical parameters, protein expression and transcript levels of E-cadherin gene. T teste was applied to evaluate the methylation statistical different in each group.

Results

Clinical Features

Thirty percent (6/20) of PC samples were Gleason score 6, 15% (3/20) score 8 and 55% (11/20) score 10. Seventy-three percent of PC samples showing Gleason score 10 had metastasis and the other 27% (3/11) showed no metastasis at the diagnosis. Dogs with PC Gleason 8 had no metastasis (n=3) and 50% (3/6) of dogs with Gleason 6 PC showed metastasis at the diagnosis. The most common metastatic sites were pelvic bones (9/11) and lungs (9/11). Eight out of 11 patients showed metastasis in pelvic bones and lungs. The other metastatic sites were intestine and liver.

We lack de clinical information of two patients (2/20). Six patients received low dose of metronomic therapy as a therapeutic option, six received only Piroxicam, in two patients performed radical prostatectomy was performed and the owners not accept any therapeutic approach in four patients. Patients

with low Gleason score (6 and 8) experienced a higher survival time (P=0.003) (Figure 1).

Immunohistochemical Features

E-cadherin showed a membranous standing in normal, PIA, PC and metastasis. Using ImageJ threshold tool, we did not find statistical difference among E-cadherin immunoexpression among all groups (normal, PIA, PC and metastasis). Normal samples showed a mean of 16.01 ±2.4E-cadherin positive cells, PIA samples had a mean of 10.84 ±2.6, PC samples had a mean of 18.7 ±6.7 and metastasis 17.5 ±5.4. PIA group showed the lowest expression levels, however, there was no statistical difference between normal and PIA or normal and PC (P>0.05).

Using the IHC percentage of negative cells (Figure 2), we found higher number of negative cells in PC samples compared to normal and PIA samples. There were no E-cadherin negative cells in normal samples; PIA samples showed a mean of 2.1% of negative cells and PC samples showed a mean of 10.5% of negative cells. Metastasis had a mean of 9.5% of negative cells. Patients showing tumors with percentage of negative cells less than 10% experienced a higher survival time (P=0.004) (Figure 3). Tumor showing Gleason score 10 had a higher number of negative E-cadherin neoplastic cells compared to PC showing Gleason Score 6 and 8 and normal samples (P=0.0003). Metastasis had a higher number of negative cells compared to normal samples (P=0.0003) and there was no statistical difference between PC and metastasis (P>0.05).

There was a positive correlation between Gleason score and the number of negative E-cadherin neoplastic cells (R= 0.8505 and P<0.0001). Thus, samples showing high Gleason score showed a higher number of neoplastic negative E-cadherin.

Western blotting

We identified a strong band with 120 Kda in normal prostate (Figure 4). There was no statistical difference in E-cadherin in normal prostate and PIA samples. However, we observed low E-cadherin protein expression in PC samples compared to normal samples (P=0.0003) and PIA samples (P=0.0001).

RT-qPCR results

We found low *CDH1*transcript levels in PC samples compared to PIA (P=0.0038) and normal samples (P=0.0427) (Figure 5). There was no statistical difference in transcript levels between PIA and normal samples. The median of *CDH1* relative quantification (RQ) in normal samples was 0.7 (0.2-9.5). PIA samples showed a median RQ of 0.9 (0.2-5.6) and PC 0.5 (0.02-1.7) and metastasis 3.45 (0.6-2.4). We identified a positive correlation between *CDH1* transcript levels and protein expression by Western blotting in PC. Thus, samples showing high transcript levels also showed higher protein expression (Spearman R=0.9429; P=0.0167) (Figure 6).

Quantitative bisulfite pyrosequencing

We identified hypermentilation of the *CDH1* promoter region in PIA and PC samples compared to normal samples (P<0.0001). The median of methylation in normal samples was 20.5% (7%-55%), in PIA samples 98% (94%-100%) and PC samples 95% (94%-100%) (Figure 7).

Tumor-derived cell cultures

We grew our two cell lines from prostate cancer samples showing *CDH1*hypermethylation, previously detected by pyrosequencing. We identified a cellular clone with homogenous epithelial cells after five passage (P5). We grew or cell lines until P10 (Figure 8) and we extract DNA from both cell lines in triplicate to evaluate the methylation status in culture condition. Although we identified hypermethylation of *CDH1* gene primary tumor samples, both cell lines showed *CDH1* demethylation in culture conditions.

The primary tumors and its cell cultures were positive for pancytokeratin and PSA and were negative for P63 and vimentin.

Discussion

After the publication of the Gleason score system in 2015, our research shows the first correlation between Gleason score and survival time in dogs. Furthermore, we found a strong correlation between the Gleason score and the number of E-cadherin negative cells. Human pancreatic adenocarcinoma with E-cadherin loss was associated with poorly differentiated tumors, having the more undifferentiated tumors the higher loss of E-cadherin expression[22]. In

human prostatic carcinoma, Ipekciet al.[23] failed to demonstrate a correlation between loss of epithelial markers, including E-cadherin and patients with higher Gleason score. In human gastric cancer, E-cadherin loss was observed in 68% of the tumors studied (34/50) by Gaoet al.[1], and was associated with worst prognosis and lymph node metastasis. An interesting finding was that the adjacent tissue also had a lower E-cadherin expression, although the authors did not mention if there was any pre neoplastic lesion in that area[1].

Hong et al[22] described two patterns of E-cadherin loss in 329 human pancreatic adenocarcinomas, and reported that 43% of the tumors (141/329) had partial (95% - 134/141) or complete (5% - 7/141) loss. Our results are similar, since we found in canine prostatic carcinoma partial loss of E-cadherin in all of our PC samples. In this research, the tumor group with Gleason score 10 had an undifferentiated pattern and showed a diffuse expression of E-cadherin. When we tried to evaluate by ImageJ program the number of E-cadherin positive cells, we did not find any statistical difference. Thus, we decide to count the number of negative E-cadherin cells in the same images as used in ImageJ analysis.

We used this percentage because E-cadherin is cell-to-cell adhesion molecule and their loss is correlated with epithelial mesenchymal transition and metastasis[7]. Loss of E-cadherin is one of the most important steps in metastasis development[23]and only few cells can be responsible for the metastatic process. We believe these negative cells are important to tumor progression and their analysis correlated with survival time, Gleason score and histological pattern. Comparing pancreatic adenocarcinomas with partial and total E-cadherin loss with tumors with intact expression, Hong et al.[22] found a

difference in survival time among these three groups, being the lowest survival time in patients with total loss of E-cadherin. They suggest that partial or total loss of E-cadherin is an independent negative prognostic factor. In human breast cancer, different authors associated E-cadherin down expression with worst prognosis, such as lower overall survival time and disease free survival and positive lymph node[24,25] and higher proliferative rate, evaluated by Ki-67[26]

In a series of 103 prostatic carcinomas, Ipekciet al.[23] did not find correlation between E-cadherin and other EMT markers with disease free survival, although patients had E-cadherin decrease expression in the tumor. E-cadherin did not demonstrate association with tumor stage, grade and volume. The authors suggest that EMT is an early event in tumor progression, so the changes in EMT proteins cannot be detected in the primary tumor, so it is not a good predictor of metastatic potential. In our study we did not have a correlation between E-cadherin negative cells and metastasis, since the number of dogs with metastatic disease was low, but we did find a correlation of lower E-cadherin expression and survival time.

Cells showing no E-cadherin expression acquire motility ability and shows an invasive phenotype and few cells without E-cadherin expression are required to develop micrometastasis[27]. Thus, the evaluation of this cell group is important to the better understanding of the metastatic process. E-cadherin downregulation occurs in most cases by posttranscriptional mechanisms[27]. The hypermethylation of the *CDH1* promoter is widely studied in many human cancers including prostate cancer[14,15,28]. The methylation as an E-cadherin regulatory mechanism permits the dynamic expression of this protein and

neoplastic cells can change their E-cadherin expression according to each step of metastatic process[5].

We investigate E-cadherin gene and protein expression in canine PC and we found a decreased expression. There was a positive correlation between E-cadherin transcript levels and protein expression, indicating that the quantity of transcript produced by neoplastic cells was translated into protein. We found *CDH1*promoter methylation in PIA and PC lesions and, using immunohistochemistry, we identified epithelial cells E-cadherin negative in both lesions.

Using the canine PC cell cultures, we expected to find *CDH1*promoterhypermethylation in these cells, since we established the cell cultures from PC samples showing hypermethylation of *CDH1* promoter. However, we found no methylation. We believe this occurred due to epigenetic instability under culture conditions. The different methods in cell culture can interfere in genetic and epigenetic stability including medium type, culture substrate, passaging method and culture supplements[29]. The prior knowledge of the*CDH1* hypermethylation in the respective primary tumors and the *CDH1* hypomethylation under culture conditions indicates a DNA promoter methylation as a regulatory mechanism of E-cadherin gene. Thus, the E-cadherin expression can change according to microenvironment conditions and contribute to PC tumorigenesis.

Conclusions

Our results suggested a downregulation of E-cadherin in canine PC and the hypermethylation of the *CDH1* promoter region can be responsible for the gene

silencing. Our score of E-cadherin negative cells correlated with higher Gleason score and lower Survival time.

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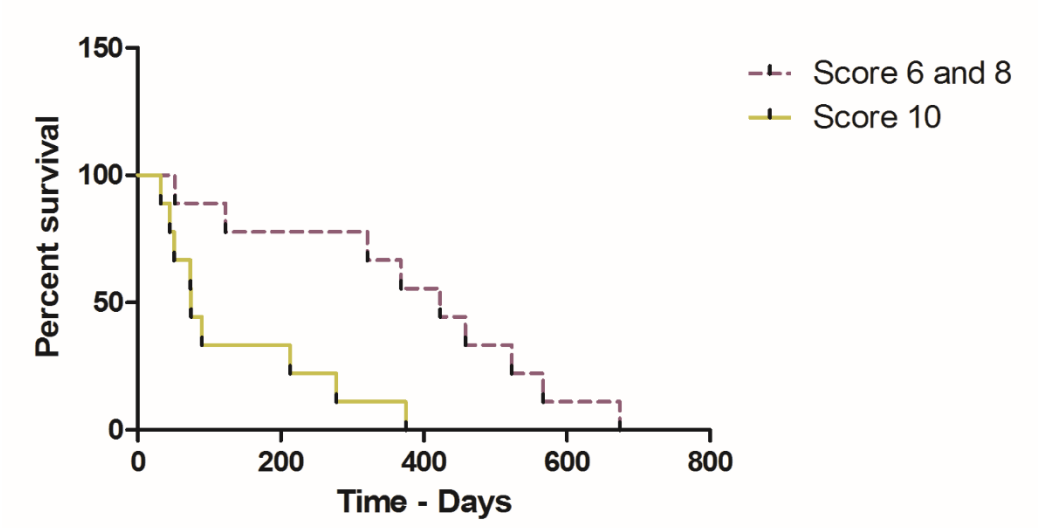

Figure 1. Overall survival of patients with prostate cancer according to Gleason score. Patients with tumors showing scores 6 and 8 experienced a higher survival time than patients showing score 10.

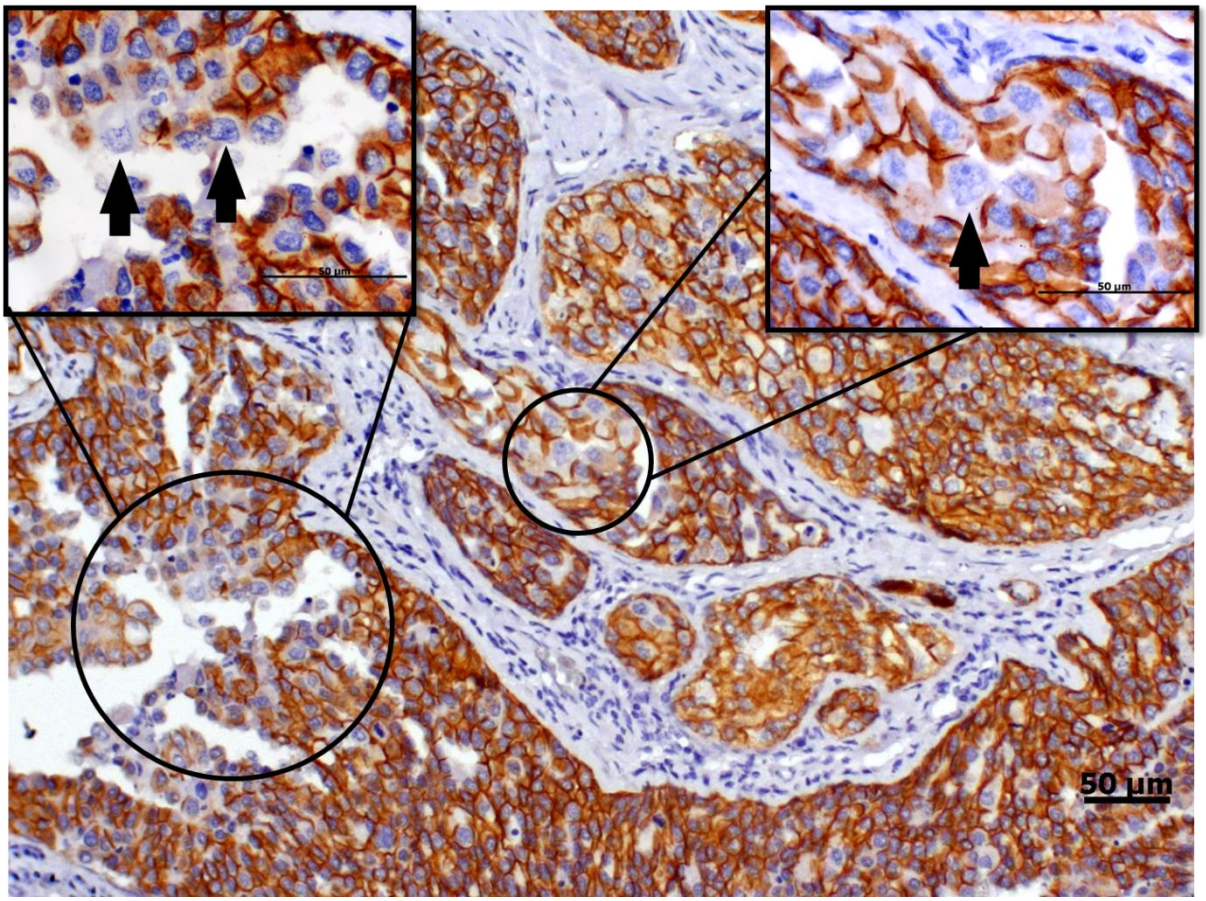

Figure 2. Immunohistochemistry of a Gleason score 10 prostate cancer, E-cadherin staining. It is possible to observe a membranous E-cadherin staining in neoplastic cells. Few neoplastic cells shows no E-cadherin expression (insert – arrows). Immunohistochemistry, peroxidase, DAC, Harris hematoxilin counter stain. (Bar - 50µm)

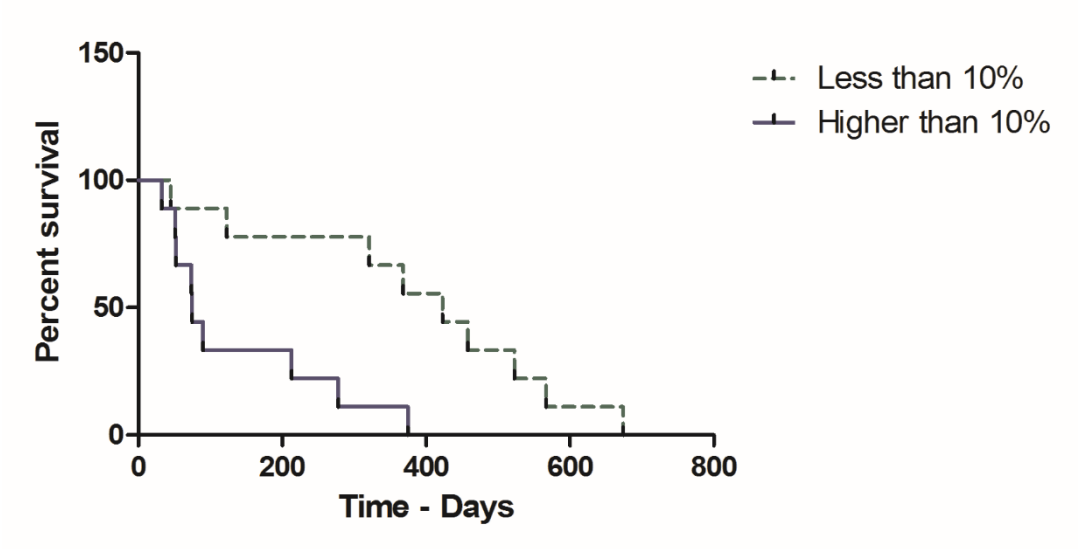

Figure 3. Overall survival of patients with prostate cancer according to negative E-cadherin cells. Patients with tumors showing less than 10% of negative cells experienced a higher survival time compared with patients showing tumors with more than 10% of cell negatives for E-cadherin (P=0.004)

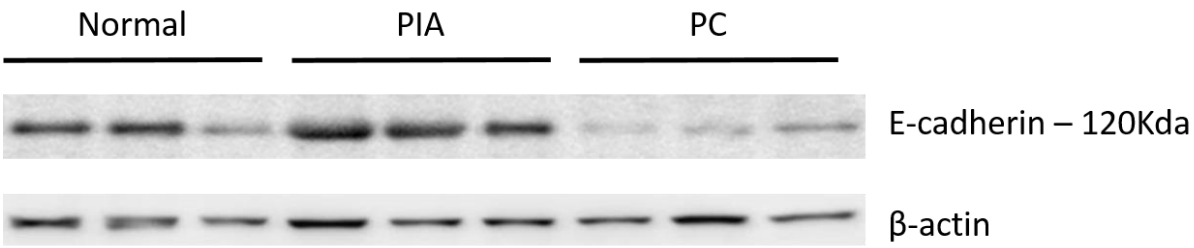

Figure 4. E-cadherin Western blotting analyzes in normal, proliferative inflammatory atrophy (PIA) and prostate cancer (PC) samples. There was no difference in E-cadherin expression between normal prostate and PIA. However, there was an E-cadherin downregulation in PC samples compared to normal and PIA.

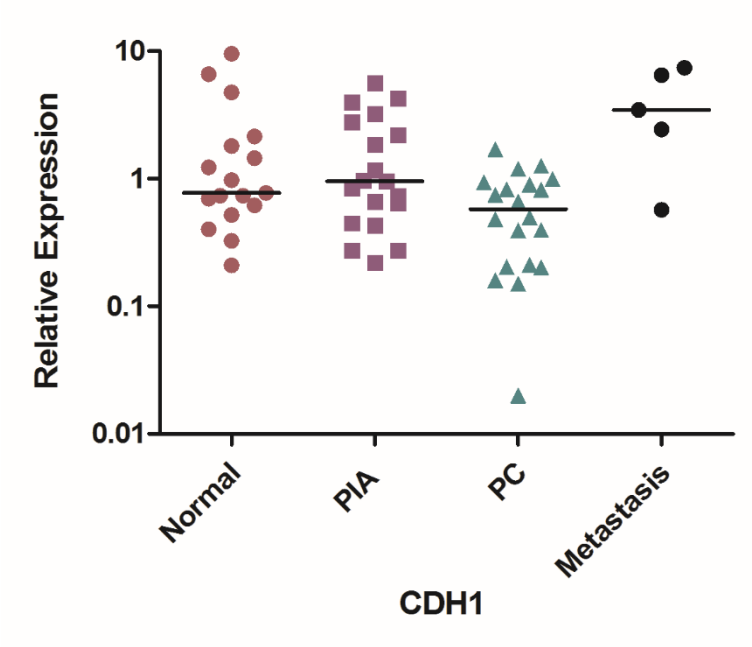

Figure 5. *CDH1* expression in canine prostatic lesions. There was no statistical difference among groups. However, prostate cancer PC) samples showed the lowest transcript levels. (PIA - proliferative inflammatory atrophy; PC – prostate cancer)

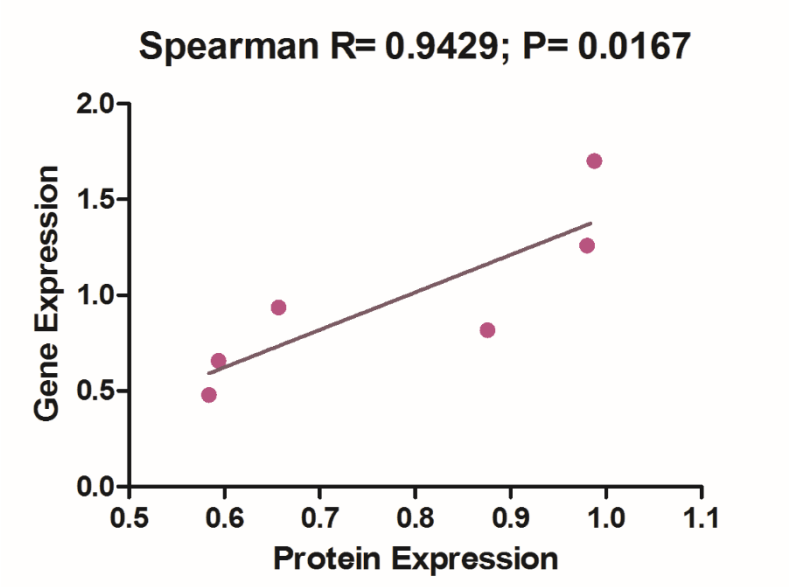

Figure 6. Positive correlation between E-cadherin gene and protein expression (by western blotting) in canine PC samples.

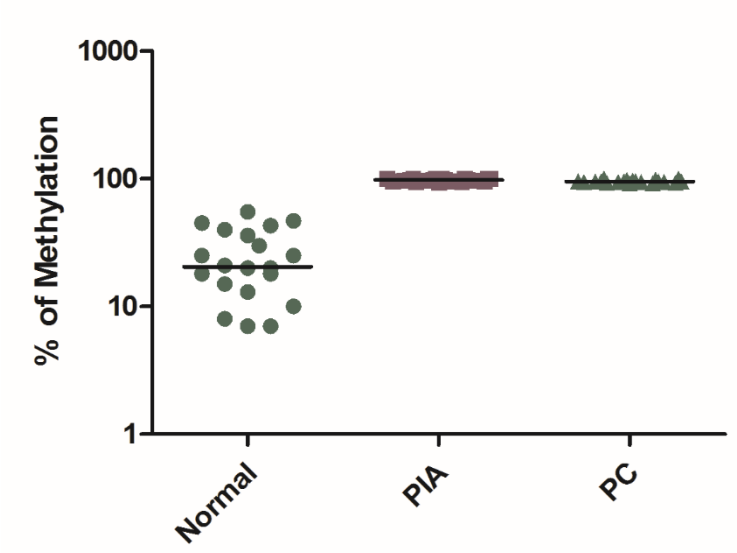

Figure 7. Graphic representation of the methylation status of *CDH1.* Proliferative inflammatory atrophy (PIA) and prostate cancer (PC) showed higher *CDH1*hypermethylationcompared to normal samples.

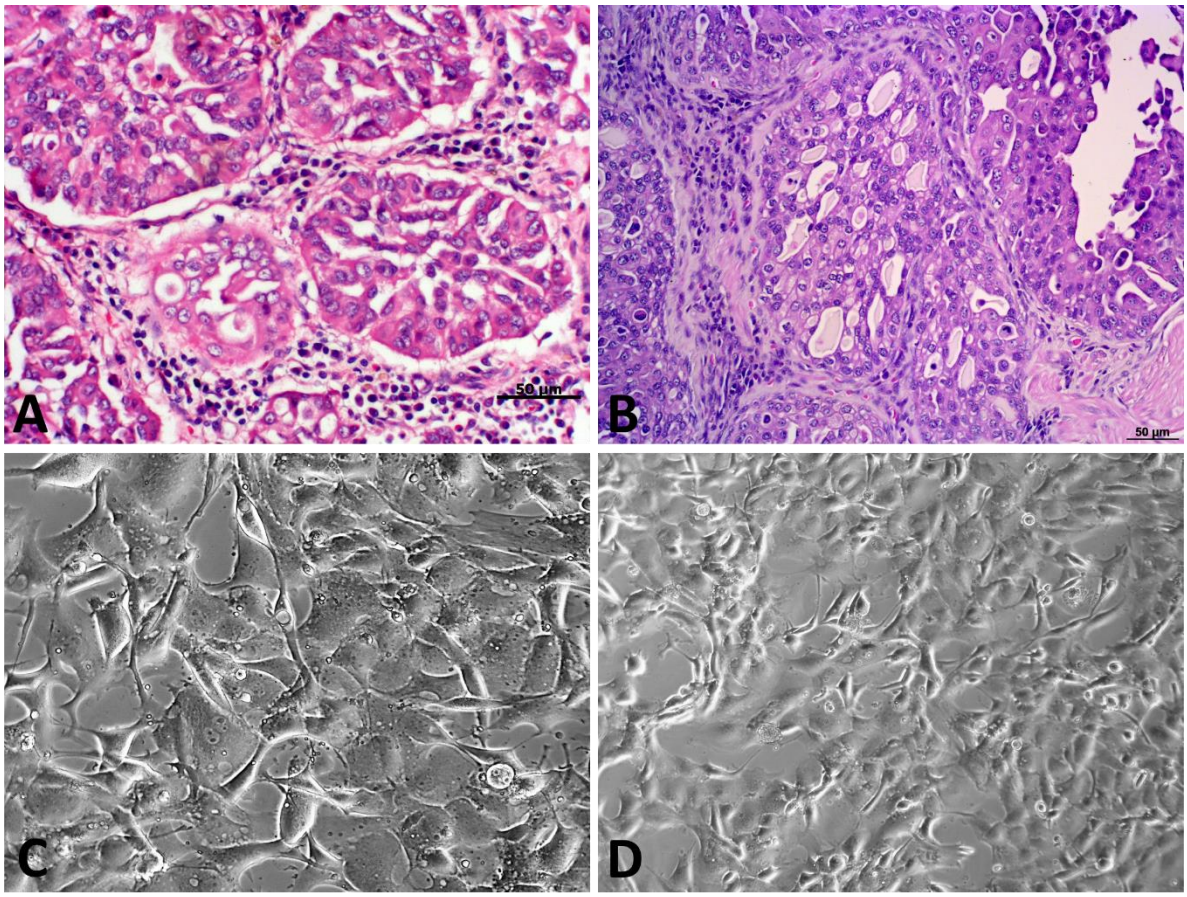

Figure 8. Canine prostate cancer (PC) used in cell culture analysis. A and B: hematoxylin and eosin (H&E) staining of the respective primary tumors. C and D: cell culture from canine PC at passage 10.

CAPÍTULO IV

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NKX3.1 downregulation and *C-MYC* overexpression are associated with canine prostate cancer progression

Abstract

NKX3.1/*C-MYC* cross regulation has been reported in human normal prostate and loss of *NKX3.1*and gain of *C-MYC* were widely reported. Dogs can be an interesting model to human prostatic disease and only one previous research had shown deregulation of NKX3.1 and MYC in canine prostate. We use prostatic samples to verify NKX3.1 and C-MYC protein and gene expression, using Western blotting and RT-qPCR. We also evaluate the NKX3.1 and MYC immunolocalization by immunohistochemistry and*NKX3.1* promoter hypermethylation. NKX3.1 showed nuclear/cytoplasmic localization in normal samples and there was no NKX3.1 expression in any canine PC samples analyzed. MYC showed low cytoplasmic expression in normal samples and PC samples showed higher nuclear/cytoplasmic expression. We identified NKX3.1 protein and gene downregulation in PC compared to normal prostate and C-MYC protein and gene overexpression in PC samples compared to normal samples. We found a correlation between NKX3.1 and MYC expression in normal and preneoplastic lesions. Thus, samples showing higher *C-MYC* expression showed higher*NKX3.1* expression indicating a *C-MYC* regulation by *NKX3.1* transcript levels. In prostate cancer, we did not find NKX3.1/MYC correlation and all PC samples showed NKX3.1 downregulation and MYC overexpression. NKX3.1 downregulation was not related to hypermethylation of their promoter in canine PC samples.

Introduction

The Consensus of the animal models of human prostate cancer describes many models to androgen-independent PC study and highlights the dog as a natural model to PC study (Ittmann et al., 2013). Dogs are the only mammal, besides humans, that spontaneous develops prostatic carcinoma (PC) with high frequency (LeRoy and Northrup, 2009). In dogs, PC is a disease with highly undifferentiated morphology and has an aggressive behavior associated with high metastatic rate (Fonseca-Alves et al., 2015). Different from human, one study had shown the aggressive behavior of canine PC and their independency to androgen stimulation in cancer initiation (Rivera-Calderón et al., 2016). Thus, dogs are relevant models to advanced human PC study, focused on pre-clinical trials and the research of new prognostic and predictive markers may benefit both species (LeRoy and Northrup, 2009).

Genes from the homeobox family appear to present a close relation with prostatic carcinogenesis (Thangapazham et al., 2014).*NKX3.1* is a homeodomain-containing transcription factor located at the human chromosome 8pandits decreased expression was associated with prostatic carcinogenic process (Asatiani et al., 2005). Many studies had shown the importance of *NKX3.1* as a suppressor tumor gene in human prostate and there is a close relation between the androgen resistance and *NKX3.1* down expression (Bowen et al., 2000; Asatiani et al., 2005; Thangapazham et al., 2014).

C-MYC oncogene is widely studied in human cancers and there is a close relation between *C-MYC* copy number gain and metastasis development in PC (Ellwood-Yen et al., 2003; Zafarana et al., 2012). Many studies had shown MYC overexpression in the prostatic intraepitelial neoplasia (PIN); however, only overexpression of MYC is not sufficient to cancer progression (Jenkins et al. 1997,Nesbit et al. 1999, Qian et al. 1997 and Sato et al. 1999). In prostate cancer, MicroRNA-34a (miR-34a) can regulate *C-MYC* oncogene, suppressing or overexpressing their transcript levels (Yamamura et al., 2012). Recently, *NKX3.1/C-MYC* cross regulation was reported (Anderson et al., 2012). In normal human and mice prostate, *NKX3.1*transcript regulates *C-MYC* preventing its overexpression. In carcinogenic process of human prostate, *NKX3.1* downregulation occurs and consequently *C-MYC* overexpression. These researchers found *NKX3.1* binding sites in *C-MYC* genes proving their

cross regulation (Anderson et al., 2012). In veterinary medicine, one previous study reported the immunoexpression of NKX3.1 and MYC in canine prostate lesions (Fonseca-Alves et al., 2013).

Due to limited information about NKX3.1 and C-MYC in canine PC and the important role of these genes and proteins in human and canine PC, our research aimed to evaluate NKX3.1 and C-MYC gene and protein expression in canine prostatic tissue.

Methods

Tissue Selection

We included in our study sixty-three canine prostatic tissue obtained during prostatectomy (surgical biopsy) or necropsy from the Veterinary Pathology archive at the University of Sao Paulo State, from 2011-2015. We selected 20 normal prostatic tissue, 20 proliferative inflammatory atrophy (PIA) lesions, 20 canine PC and three metastasis. PC samples that were collected during prostatectomy surgery or biopsies procedure were from animals showing clinical signs and complementary exams indicating a neoplastic process. Normal samples were collected during necropsy from animals without clinical signs of prostatic disease. All prostate samples were from intact dogs. From PC subjects, clinical records were assessed to obtain the patient information, treatment modalities and outcome for each patient. All samples were formalin fixed and paraffin embedded (FFPE) and histological classification was done according to Lai et al. (2008) and the Gleason-like score according to Palmieri and Grieco (2015). For Western blotting, frozen samples were also collected and kept in a minus 80°C ultra freezer.

Immunohistochemistry

We performed immunohistochemical evaluation in four normal sample, four PIA, four and three metastasis to show the immunolocalization of each antibody in normal and PC samples. Slide sections were dewaxed in xylene and rehydrated in graded ethanol. For antigen retrieval, the slides were incubated with citrate buffer (pH 6.0) in a pressure cooker (Pascal®; Dako, Carpinteria, CA, USA). The slides were treated with freshly prepared 3% hydrogen peroxide in methanol for 20 min to inhibit endogenous peroxidase activity and further washed in Tris-buffered saline. The primary antibodies were diluted in 1:50 to NKX3.1 and 1:300 to MYC and the slides were incubated over night at 4^0C. A polymer system (Envision, Dako, Carpinteria, CA, USA) was applied as a secondary antibody conjugated to peroxidase and 3´-diaminobenzidine tetrahydrochloride (DAB1, Dako, Carpinteria, CA, USA) was used as the chromogen, for 5 min, followed by Harris hematoxylin counterstain.

After each step of the immunohistochemical process, the slides were rinsed with Tris-buffered saline. Negative controls were performed for all antibodies by omitting the primary antibody and replacing them with Tris-buffered saline. Membranous, cytoplasmic or nuclear immunolocalization was recorded for each antibody.

Western Blotting

We performed Western blotting in six normal prostate, six PIA lesions and six PC to evaluate MYC and NKX3.1 protein expression. The frozen prostate samples were cut in a cryostat to confirm the previous diagnosis and then were mechanically homogenized in 50 mMTris–HCl buffer pH 7.5, 0.25%

Triton X-100 and EDTA by Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 30s at 4°C. Equal amounts of protein (70µg) obtained from the samples were heated at 95°C for 5 minutes in the sample-loading buffer and were then subjected to SDS–PAGE separation or electrophoresis under reducing conditions and were transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO).

The blots were blocked with 6% skimmed milk in TBS-T (10 mMTris–HCl pH 7.5, 150 mMNaCl, 0.1% Tween-20) for two hours and the anti-NKX3.1 (1:300) and anti-MYC (1:800) antibodies were incubated for 18 hours (overnight). Goat anti-β-actin antibody (1:1:000; sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a positive 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). Protein bands were quantified by densitometry analysis and expressed as integrated optical density (IOD). NKX3.1 and MYC protein expression were normalized to the β-actin values. Normalized data are expressed as the means and standard deviation (SD).

Gene expression

The paraffin-embedded tissue samples were macrodissected using 16 gauge needles and mRNA were extracted using a commercial RecoverAll™ Total Nucleic Acid Kit (Ambion, Life Technologies, MA, USA) according to the manufacturer’s instructions. The mRNA concentration was determined with a

spectrophotometer (NanoDrop™, ND-8000, Thermo Scientific, MA, USA), while the mRNA 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 50ºC, and the enzyme was subsequently inactivated for 15 min at 70ºC. cDNA was stored at -80ºC.

RT-qPCR for *NKX3.1* (Forward: 5'-TGAGGTGGTTGGAGGTTTGC-3' and Reverse: 5'-TTTCATTGGCCCATCACTGA-3')and *C-MYC*(Forward: 5'-GCTGCCGCTGTCACTATGG-3' and Reverse: 5'-GAACTGCTCGCGCTTCGA-3') and the endogenous genes *HPRT* (Forward: 5'-AGCTTGCTGGTGAAAAGGAC-3' and Reverse: 5'-TTATAGTCAAGGGCATATCC-3'), *ACTB*(Forward: 5'-GGCATCCTGACCCTCAAGTA-3' and Reverse: 5'-CTTCTCCATGTCGTCCCAGT-3') and *PRS5* (Forward: 5'-TCACTGGTGAGAACCCCCT-3' and Reverse: 5'-CCTGATTCACACGGCGTAG-3') were 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. Relative gene expression was quantified using the 2-ΔΔCT method.

Quantitative bisulfite pyrosequencing

The pyrosequencing analysis was performed to*NKX3.1* gene to evaluate the frequency of methylation in their promoter. We used 20 normal prostates, 20 PIA samples and 20 PC. The frozen prostate samples were cut in a cryostat to confirm the previous diagnosis. We performed the bisulfite conversion of the genomic DNA using EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Irvine, CA, USA). We performed a forward primer (5'GGGATTTGTGTTTTTTGT 3') and reverse primer (5'ACTAATCAAAACCCCATC - BIOTIN 3') from *NKX3.1*CpG islands and amplified using PCR (HotStarTaq Master Mix kit - Qiagen). The pyrosequencing was performed using a sequencing primer (5' GAATTAGTTGGAGA 3') following the manufacturer's instructions (PyroMark ID Q96, Qiagen and Biotage, Uppsala, Sweden).

Statistical analysis

To qRT-PCR, normal, PIA, PC and metastasis group were evaluated as the median and ANOVA test or T test was applied to verify statistical difference in gene expression among groups. Survival curve was calculated only for PC using the Kaplan-Meier method, and statistical significance was determined using a log-rank test. Overall survival was defined as the period (in months) between the date of surgery and death caused by the disease. T test was used for Western blotting analysis. The Kruskal-Wallis or Mann-Whitney U test was

applied to analyze *NKX3.1 and C-MYC* transcript levels. *P*<0.05 was considered significant for all variables using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

Results

Clinical data

Thirty percent (6/20) of PC samples showed Gleason score 6, 15% (3/20) had Gleason score 8 and 55% (11/20) had Gleason score 10. Seventy-three percent of PC samples showing Gleason score 10 had metastasis at the diagnosis. Gleason 8 samples had no metastasis (n=3) and 50% (3/6) of Gleason 6 samples showed metastasis at the diagnosis. The most common metastatic site were pelvic bones (9/11) and/or lungs (9/11). Eight out of 11 patients showed metastasis in pelvic bones and lungs. The other metastatic sites were intestine and liver.

We lack the clinical information of two patients (2/20). Six patients received low dose of metronomic therapy as a therapeutic option, six received only Piroxicam, in two patients was performed radical prostatectomy and the owners not accept any therapeutic approach in four patients. Patients with lower Gleason score (6 and 8) experienced a higher survival time (P=0.003) than Gleason 10 patients.

Immunohistochemistry

We identified a nuclear/cytoplasmic NKX3.1 expression in normal (Figure 1A) and PIA samples. The NKX3.1 expression was restricted to luminal epithelial cells. There was no NKX3.1 expression in basal or stromal cells. The four PC samples analyzed showed no NKX3.1 expression (Figure 1). All metastasis were negative for NKX3.1 staining.

MYC antibody showed weak cytoplasmic expression in normal samples (Figure 2) and nuclear/cytoplasmic expression in PIA samples. All PC samples and metastasis showed a strong nuclear and weak MYC cytoplasmic expression (Figure 2).

Western blotting

We identified a band with 26 KDa to NKX3.1 protein. We observed NKX3.1 down-regulation in PC samples compared to normal and PIA samples (P=0.0018). There was no statistical difference between normal and PIA lesions. However, there was decrease expression in PC compared to normal samples (P=0.0012) and PIA samples (P=0.001) (Figure 2).

MYC showed a 50Kdaband in western blotting analysis in all prostatic tissues (Figure 3). We observed a higher MYC expression in PC samples compared to normal (P=0.001) and PIA samples (P=0.003). We identified a positive correlation between NKX3.1 and MYC expression in normal and PIA samples. Thus, samples showing higher MYC protein expression showed the highest NKX3.1 expression. We did not find this correlation in PC group. In PC samples, all tumor showed high MYC protein expression and low NKX3.1 protein expression.

Quantitative real time PCR

We identified a very interesting *NKX3.1* results. There was a gradual decreasing *NKX3.1* expression among normal, PIA, PC and metastasis samples. Where normal samples showed the highest transcript levels and metastasis showed the lower transcript levels (Figure 4). We identified a positive correlation between *NKX3.1* gene and protein expression (Spearman R: 0.7143) in normal and PIA samples; however there was no correlation in PC samples.

As opposite to *NKX3.1*, we identified a gradual increased *C-MYC* expression among, normal, PIA, PC and metastasis. Normal samples showed the lowest transcript levels and metastasis showed de highest transcript levels (Figure 5). We found a positive correlation between C-MYC gene and protein expression. Thus, samples showing the highest transcript levels also had the highest protein levels. In PC samples, there was no correlation between *C-MYC*

gene and protein expression. We performed the correlation between the *NKX3.1* and *C-MYC* transcript levels in normal, PIA and PC samples. Interesting, we found a positive correlation between *NKX3.1* and *C-MYC* transcript levels in normal (Spearman R: 0.6494) and PIA (Spearman R: 0.3725) samples. However, in PC samples we found a negative correlation between *NKX3.1* and *C-MYC* transcript levels (Spearman R: -0.6630). Thus, samples showing highest *C-MYC* expression showed the lowest *NKX3.1* expression.

Quantitative bisulfite pyrosequencing

We investigate*NKX3.1* promoter hypermethylation due gene and protein decreased expression. We did not find statistical difference in methylation analyses among normal, PIA and PC samples (Figure 6). However, three samples of canine PC had *NKX3.1* promoter hypermethylation higher than 20%.

Discussion

We found NKX3.1 downregulation in all PC samples indicating that NKX3.1 loss is important to PC development, same as we reported previously (Fonseca-Alves et al., 2013). Interestingly, we identified a decreased in *NKX3.1* transcript levels among different prostatic lesions and our results indicated a role of *NKX3.1* in cancer progression and metastasis. PIA samples showed a lower *NKX3.1* transcript levels compared to normal samples and there was a more evident loss in PC and metastatic samples, which showed de lowest transcript levels. We evaluated three respective primary tumor and their metastasis and the metastatic tumors showed lowest *NKX3.1* transcript levels compared to others primary tumors.

In human prostate cancer, loss of NKX3.1 occurs only in advanced prostate cancer (Asatiani et al., 2005). Most of the PC cases in humans shown positive NKX3.1 expression and its loss was associated with poor prognosis (Bowen et al., 2000). It is a consensus in the literature that canine PC is more aggressive than the human counterpart. The NKX3.1 downregulation we found can interfere with the aggressive behavior of canine PC.

Deletion in 8p21.2 region and hypermethylation of the *NKX3.1* promoter has been reported in human PC as mechanism of *NKX3.1* downregulation in up to 85% of cases (Asatiani et al., 2005). In dogs, our research group investigated *NKX3.1* deletion in canine PC; however, we did not find any alteration in *NKX3.1*using aCGH (data not shown). Thus, we believed that promoter hypermethylation could be a regulatory mechanism for *NKX3.1* in canine PC. Our methylation results did not identify hypermethylation of the *NKX3.1* promoter. Only three cases (3/14) showed promoter methylation higher than 25% and lower than 30%.

Our results showed C-MYC overexpression (gene and protein) in canine PC compared to normal and PIA samples. We performed a correlation between *C-MYC* and *NKX3.1* gene and protein expression and we found a positive correlation in normal samples. Anderson et al. (2012) reported a cross regulation between *NKX3.1* and *C-MYC*. These authors looked at *NKX3.1* target genes and identified a fraction of *NKX3.1* gene that is a direct *C-MYC* oncoprotein target. Thus, *NKX3.*1opposes the transcriptional C-*MYC* activity. Furthermore, *NKX3.1* downregulation may contribute to MYC overexpression in cancer progression (Anderson et al., 2012). In our study, our correlation results in normal samples indicate a crossregulation between *NKX3.1* and *C-MYC* in accordance with Anderson et al. (2012). However, in PC samples we identified a negative *NKX3.1*/*C-MYC* correlation, indicating *NKX3.1*as a target to C-MYC transcriptional activity and *NKX3.1* downregulation can contribute to *C-MYC* overexpression and cancer progression.

Our results indicated a gradual *C-MYC* increased expression and normal samples showed the lower transcript levels indicating a *C-MYC* role in cancer progression. We identified a positive correlation of C-MYC gene and protein expression in normal prostatic tissue and pre neoplastic lesion (PIA). However, there was no correlation between *C-MYC* transcript levels and protein expression in canine PC. This result indicates a post-transcriptional regulatory mechanism to MYC protein and the absence of correlation indicates an alternative mechanism interfering in protein translation and also a complex gene and protein relation in cancer, related to different mechanisms such as miRNAs, and pathways interactions. In humans, the regulation of C-MYC transcript by microRNA have been reported (Misso et al., 2014).

Conclusion

Our results strongly suggest a correlation between *NKX3.1* and *C-MYC* transcriptional activity and *NKX3.1*downregulation correlates with *C-MYC* overexpression in canine PC. We found a gradual decreased in NKX3.1 expression and a gradual increased in MYC expression in canine PC, indicating a role of these proteins in cancer progression. *NKX3.1* downregulation and *C-MYC* overexpression can be related with metastatic process of canine prostate.

Acknowledgment

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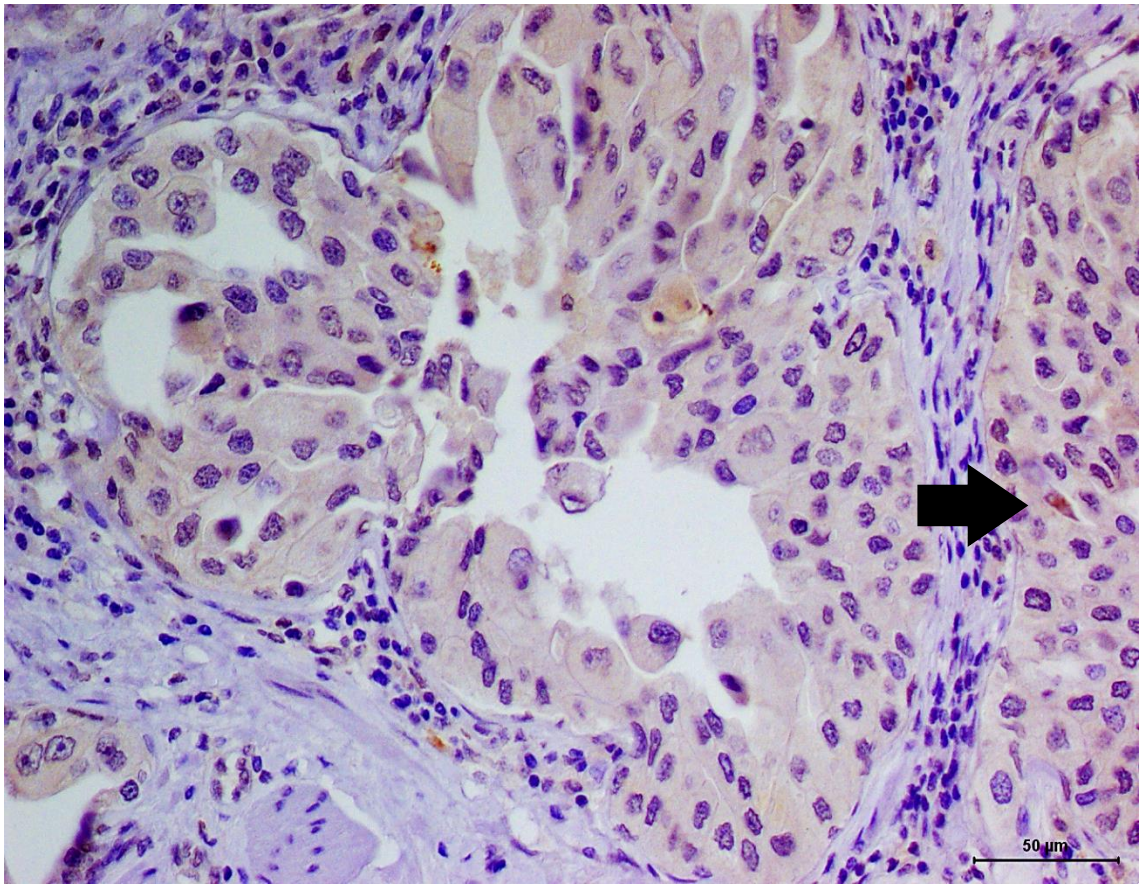

Figure 1. Immunohistochemistry for NKX3.1 protein in a canine PC. It is possible to observe cells showing cytoplasmic NKX3.1 expression and few with nuclear expression (arrow

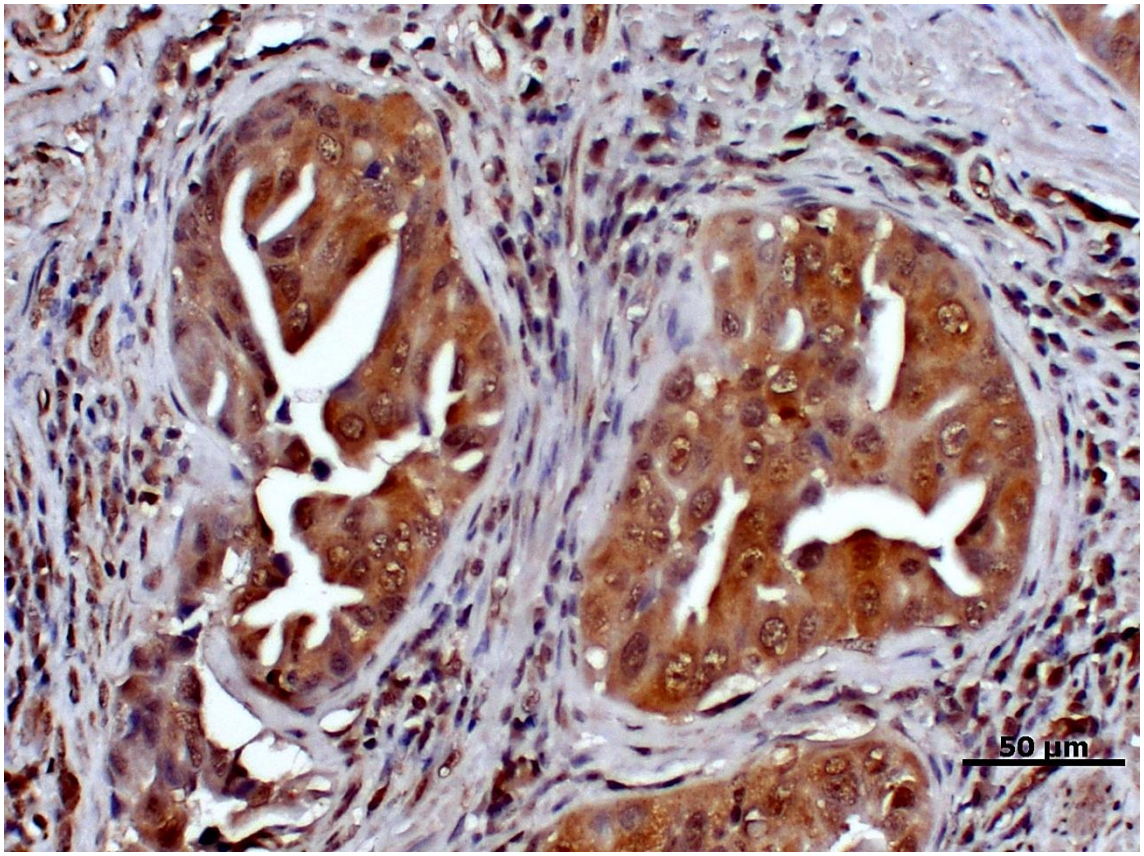

Figure 2. Immunohistochemistry for MYC in canine prostate cancer (PC). It is possible to note a strong cytoplasmic/nuclear MYC expression in neoplastic cells and in some inflammatory cells.

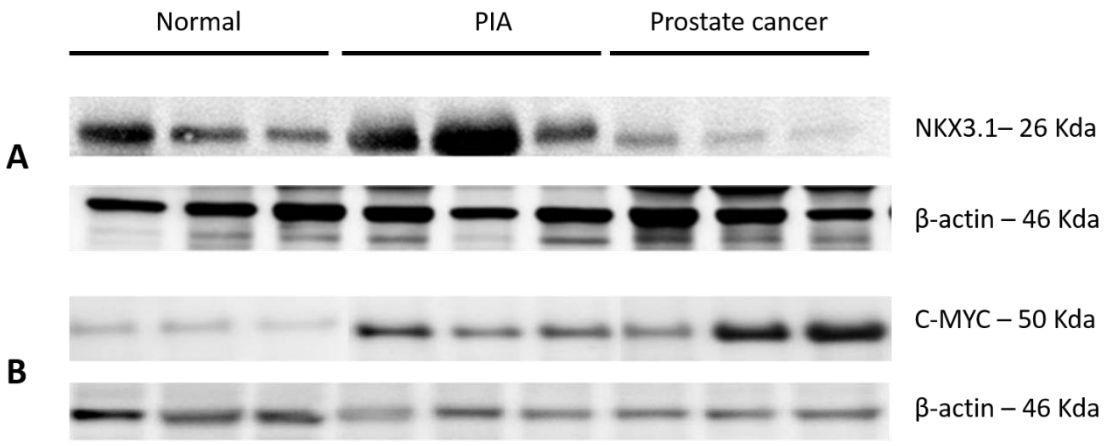

Figure 3. Western blotting analysis of NKX3.1 and MYC proteins. A: Note the NKX3.1 downregulation compared to normal and PIA samples. B: It is possible

to observe low MYC expression in canine normal tissue and an increased expression in canine prostate cancer.

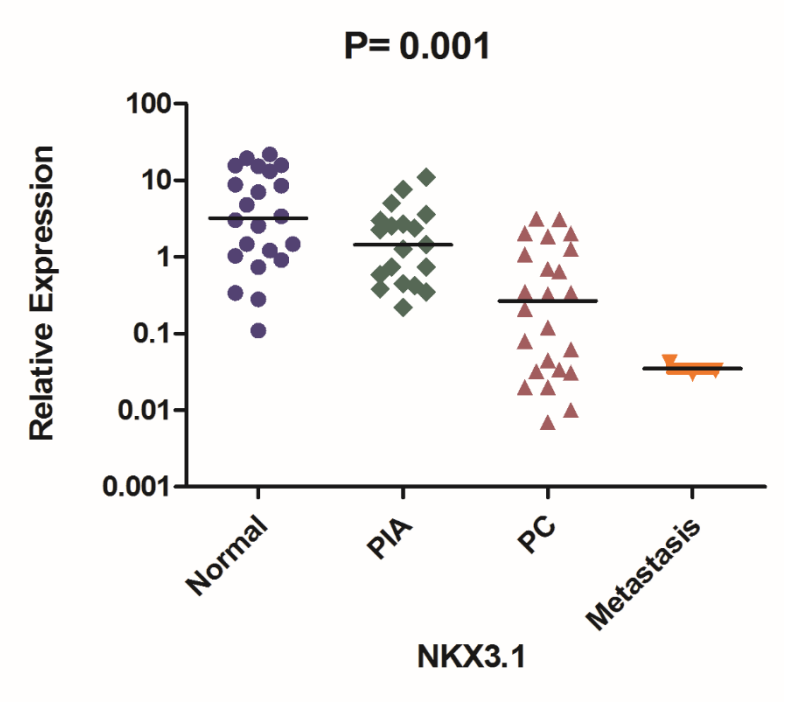

Figure 4. Evaluation of *NKX3.1* transcript levels in canine prostate. It is possible to observe a gradual *NKX3.1* decreased expression and that metastasis had the lowest transcript levels.

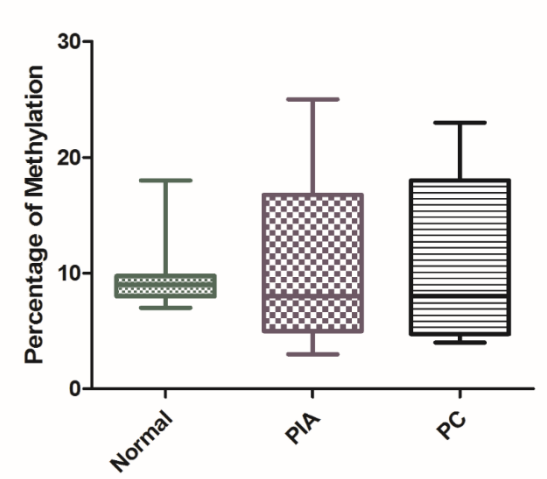

Figure 5. Evaluation of *C-MYC* transcript levels in canine prostatic tissue. It is possible to observe a gradual *C-MYC* increased among normal, PIA, PC and metastasis. Metastasis samples had the higher transcript levels.

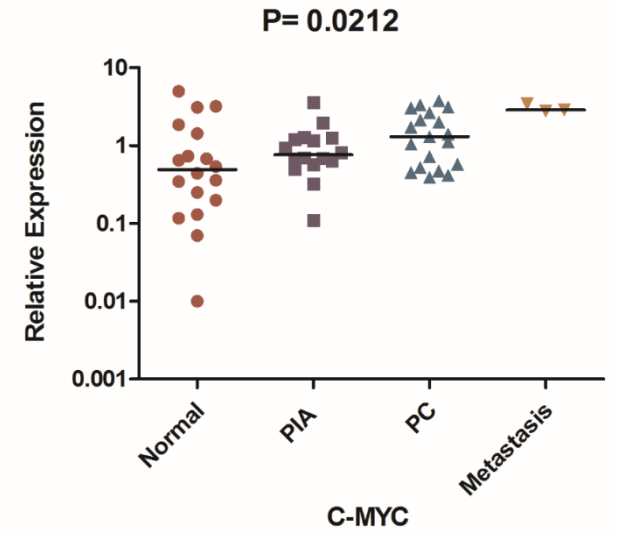

Figure 6. Evaluation of the *NKX3.1* promoter methylation in canine prostatic tissue. Normal, PIA and PC samples showed similar levels of methylation.

CONSIDERAÇÕES FINAIS

Considerações Finais

Os carcinomas prostáticos caninos são neoplasias com comportamento biológico agressivo e, ao diagnóstico, a maioria dos pacientes apresenta metástase. Esse comportamento difere do homem, que apresenta em sua maioria, carcinomas de baixo grau. A próstata canina apresenta algumas particularidades histológicas, que devem ser consideradas ao utilizar o cão como modelo de estudo para doença humana. Destaca-se a descontinuidade da camada de células basais como a principal diferença entre as duas espécies. A atrofia inflamatória proliferativa (PIA) é uma lesão com alta incidência em cães e apresentar um alto potencial como lesão pré-neoplásica da prostática canina, assim como no homem.

O escore de avaliação utilizando a porcentagem de células negativas para o anticorpo E-caderina correlacionou-se com o prognóstico dos pacientes, bem como o escore de *Gleason*. Assim, para tumores que apresentam perda focal de E-caderina, este sistema pode ser útil na avaliação destes tumores, e demonstra a heterogeneidade entre as células de um mesmo tumor. A perda de NKX3.1 é frequente nos carcinomas prostáticos caninos e esta alteração pode ser relacionada com a agressividade dos tumores em cães.

As neoplasias prostáticas caninas podem ser utilizadas como modelo de estudo para os carcinomas em humanos, no entanto, deve-se ficar atento as particularidades de cada espécie para uma melhor aplicação desse modelo em oncologia comparada.

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