UNIVERSIDADE ESTADUAL PAULISTA ”JULIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

AVALIAÇÃO MORFOLÓGICA, CELULAR E IMUNO-HISTOQUÍMICA DO CARCINOMA DE CÉLULAS TRANSICIONAIS DA BEXIGA DE CÃES

VERÔNICA MOLLICA GOVONI

BOTUCATU-SP
2021

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Tese apresentada ao Programa de Biotecnologia Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista Julio de Mesquita Filho – Campus de Botucatu, para a obtenção do título de Doutora

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Co-orientadora (Brasil): Prof. Dra. Juliany Gomes Quitzan
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DEDICATÓRIA

Dedico esta tese de doutorado à minha querida avó Suzana Maria de Tolosa Mollica, minha torcedora árdua e presente nas minhas mais doces lembranças (*in memoriam*).

EPÍFRAGE

"Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se não o fizerem ali?"

Fernando Pessoa

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Ao Prof. Dr. Carlos Eduardo Fonseca Alves, por me ter aceitado de braços abertos como sua orientada, me ajudando desde o início do meu projeto não apenas em sua execução como na gestão de novas ideias e na resolução de problemas, sempre de maneira muito solícita e paciente. Além disso, me permitiu o contato com a professora Valeria Grieco, possibilitando meu doutorado sanduíche.

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Enfim, a todos aqueles que de alguma forma colaboraram, participaram ou torceram por mim e pelo trabalho.

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RESUMO

GOVONI, V. M. **Avaliação morfológica, celular e imuno-histoquímica do carcinoma de células transicionais da bexiga de cães** [Morphological, cellular and immunohistochemical evaluation of transitional cell carcinoma of the bladder of dogs] 2021. 71 f. Tese (Doutorado em Biotcnologia Animal) - Faculdade de Medicina veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, 2021.

O carcinoma de células transicionais canino (CCT) é considerado o tumor maligno mais comum do trato urinário em cães. Devido à sua localização anatômica e progressão da doença no momento do diagnóstico, o tratamento desse câncer representa um desafio para a medicina veterinária, sendo o cão um modelo natural para a doença em humanos. O conhecimento sobre o CCT canino é limitado, portanto, o objetivo do presente estudo é realizar uma caracterização clínica, morfológica e imuno-histoquímica deste tumor. Dados clínicos de um total de 32 amostras da Universidade Estadual Paulista, do Laboratório VetPat e da Universidade de Milão (Itália) obtidas entre janeiro de 2000 e junho de 2020 foram utilizados para as avaliações clinico-patológica e imunohistoquímica dos marcadores Ki67, GATA-3 e Caveolina-1. Os fatores clínico-patológicos que apresentaram valor prognóstico no primeiro trabalho científico foram o tipo de tratamento e a invasão linfática. E o segundo trabalho cietífico demonstrou que os biomarcadores GATA-e e Caveolina-1 assim como as variáveis invasão linfática, tipo e grau histológico são potenciais fatores preditores do comportamento do CCT canino.

Palavras-chave: carcinoma urotelial, cão, oncologia, trato urinário

ABSTRACT

GOVONI, V. M. **Morphological, cellular and immunohistochemical evaluation of transitional cell carcinoma of the bladder of dogs** [Avaliação morfológica, celular e imuno-histoquímica do carcinoma de células transicionais da bexiga de cães] 2020. 71 f. Tese (Doutorado em Biotcnologia Animal) - Faculdade de Medicina veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, 2021.

Canine transitional cell carcinoma (TCC) is considered the most common malignant tumor of the urinary tract in dogs. Due to its anatomical location and disease progression at the time of diagnosis, the treatment of this cancer represents a challenge for veterinary medicine, with the dog being a natural model for the disease in humans. The knowledge about canine TCC is limited, therefore, the aim of the present study is to perform a clinical, morphological and immunohistochemical characterization of this tumor. Clinical data from a total of 32 samples from the Universidade Estadual Paulista, VetPat Laboratory and University of Milan (Italy) obtained between January 2000 and June 2020 were used for the clinicopathological and immunohistochemical evaluations of Ki67, GATA-3 markers and Caveolin-1. The clinicopathological factors that showed prognostic value in the first scientific work were the type of treatment and lymphatic invasion. And the second scientific work demonstrated that the biomarkers GATA-e and Caveolin-1, as well as the variables lymphatic invasion, histological type and histological grade are potential predictors of canine TCC behavior.

Key- words: urothelial carcinoma, dog, oncology, urinary tract

Capítulo 1

1 INTRODUÇÃO E JUSTIFICATIVA

"Em certo sentido, a doença só passa a existir quando decidimos de comum acordo que ela existe ---- percebendo-a, dando-lhe nome e respondendo a ela."
C. E. Rosenberg

Está foi uma das frases citadas por Siddharta Mukherjee, médico oncologista, em seu livro "O Imperador de Todos os Males, uma biografia do câncer" (1). Siddartha faz uma abordagem histórica, biológica e filosófica da doença que mais assusta a humanidade, e que transposta à medicina veterinária não é menos desafiadora. Foi por volta de 400 a.C. que surgiu o primeiro termo na literatura médica, *Karkinos,* de "caranguejo" em grego, símbolo hoje popularmente conhecido. O significado deste termo foi interpretado diversas vezes e de diversos modos e citado pelo autor: "o tumor, com vasos sanguíneos inchados à sua volta, fez Hipócrates pensar num caranguejo enterrado na areia com as patas abertas em círculo". E na visão de pacientes, "a súbita pontada de dor produzida pela doença era como se estivessem presos nas tenazes de um caranguejo." À diferença da medicina veterinária, porém, o paciente humano é descrito como um contador de histórias, um narrador que relata seus sintomas, que denuncia a doença. O paciente que não fala, e que por vezes disfarça sofrimento, traz aos médicos veterinários um desafio a mais na investigação do câncer. Perceber a doença é um ponto de partida difícil, mas necessário. E dar-lhe um nome e responder a ela tem sido cada vez mais possível com a evolução da oncologia, de suas técnicas diagnósticas e terapêuticas, e do estudo de seus fatores prognósticos.

O CCT da bexiga é uma das neoplasias mais desafiadoras, que em cães é comumente diagnosticada tardiamente. Além do grau avançado de evolução da doença ao diagnóstico, sua localização no órgão pode dificultar ainda mais o tratamento e seu prognóstico costuma ser pobre, com baixa sobrevida após o diagnóstico (2).

Em um paralelo histórico, entre 1891 e 1907, estudiosos provaram que grandes cirurgias eram factíveis para a remoção de tumores mamários; e realmente, o poder de extrair uma massa maligna de um órgão é a primeira escolha para a maioria dos casos de cânceres. Porém, a remoção total de um tumor, como é o caso do CCT canino, pode ser incompatível com a funcionalidade ou mesmo com a manutenção de determinado órgão, nos sendo obrigada uma avaliação racional e cada vez mais personalizada para cada paciente. A batalha contra o câncer não é uma batalha a ser vencida a qualquer custo, e sim uma batalha a favor da qualidade de vida.

Tumores localizados no trígono vesical são frequentemente irressecáveis, ou ressecáveis somente se associados a técnicas radicais como cistectomia total e reimplantações ou derivações ureterais. Tais técnicas apresentam consequências como pielonefrites recorrentes, deiscências de pontos com uroabdome, que frequentemente culminam em uma menor sobrevida. E este fato tem estimulado cada vez mais a pesquisa de novas opções terapêuticas, clínicas complementares, sistêmicas ou locais, e mais específicas.

Como relatado no livro de Siddharta, antes de 1980 todas as drogas envolvidas na terapia do câncer se baseavam em duas vulnerabilidades desta doença: o fato da doença iniciar localmente antes de se espalhar, o que permite cirurgia ou radioterapia, e o fato das células cancerosas apresentarem um rápido crescimento celular. Porém este último é alvo de fármacos citotóxicos que atingem também células normais, e causam a maioria dos efeitos colaterais. Foi visto que, embora tais fármacos sejam ainda muito

efetivos e muito utilizados, protocolos quimioterápicos radicais podem não ser a melhor opção, e novas moléculas-alvo podem e devem ser descobertas visando o ataque de novas vulnerabilidades da célula tumoral. Bruce Chabner já disse:

"A terapia perfeita não foi desenvolvida. A maioria de nós acredita que ela não terá nada a ver com terapia citotóxica, e é por isso que apoiamos as investigações básicas voltadas para uma compreensão mais fundamental da biologia do tumor [...] precisamos fazer o melhor uso possível do que temos agora."

Além disso, para o direcionamento do desenvolvimento de novas e tão almejadas terapias, é preciso saber quais os principais fatores prognósticos de um tumor e então onde atuar. Poucos estudos veterinários avaliaram o valor prognóstico de fatores associados ao CCT canino (3-6). A maioria deles estuda o efeito de algum tratamento específico como um tipo de cirurgia a ser realizado (5), ou estuda fatores clínicos intrínsecos ao tumor como a sua localização dentro da bexiga (6). Um trabalho de 2000 foi dos poucos a investigar os fatores epidemiológicos e histopatológicos deste tumor em cães como possíveis influenciadores do tempo de sobrevida, e isto em apenas 25 animais dos Estados Unidos (2). E ainda, em metanálise recente, foram revisados diversos artigos humanos e veterinários quanto a genes e proteínas expressas no CCT, evidenciando a escassez de dados veterinários de sobrevida relacionados à doença, assim como a falta frequente de informações clinico-patológicas como a presença de invasão da camada muscular (7). Em medicina humana tais dados são constantemente apresentados e permitem que o estudo de fatores prognósticos referentes a este tumor seja mais consistente.

Desta forma, considerando o conhecimento relativamente pequeno sobre o carcinoma de células transicionais no cão, o presente estudo visa avaliar amostras de CCT da bexiga de cães do ponto de vista clínico-patológico e imunofenotípico, correlacionando marcadores imuno-histoquímicos pouco ou nunca antes estudados em

medicina veterinária com variáveis clínico-patológicas, e quando possível, investigando o valor prognóstico dos mesmos.

2 REVISÃO DE LITERATURA

2.1 O carcinoma de células de transição (CCT)

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1 INTRODUÇÃO E JUSTIFICATIVA

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2 REVISÃO DE LITERATURA

2.1 O carcinoma de células de transição (CCT)

O CCT corresponde a apenas 2% dos cânceres em cães, no entanto é o mais comum do trato urinário nesta espécie. Acomete frequentemente a região do trígono vesical e tem em sua maioria caráter invasivo, podendo envolver também uretra e próstata (8-11). Em humanos, o câncer de bexiga é o nono tumor maligno mais comum, ocupando, nos países desenvolvidos, o quarto e o décimo quarto lugares em frequência em homens e mulheres, respectivamente (12).

Baseada na classificação humana, o CCT em cães foi dividido segundo a Organização Mundial de Saúde em 4 tipos histológicos principais: carcinoma não papilar e não infiltrativo; carcinoma papilar e não infiltrativo; carcinoma papilar infiltrativo e carcinoma não papilar infiltrativo (13). E embora a graduação tumoral não seja frequentemente utilizada na prática clínica, podem ser variavelmente graduados histologicamente de I a III de acordo com os critérios de Valli et al., 1995 (9) ou

Meuten et al. (2004) (14); e de I a IV pela graduação humana de Cheng, MacLennan e Lopez-Beltran, 2012 (15).

Existe uma grande dificuldade no tratamento deste tumor na medicina veterinária devido a fatores como a sua localização anatômica e o grau de evolução da doença no momento do diagnóstico (2). Em muitos casos a ressecção cirúrgica não é possível, apesar de apresentar bons resultados em termos de sobrevida dos animais (16, 17). Desta forma, o controle tumoral local costuma ser um desafio aos veterinários, uma vez que a principal causa de óbito são obstruções do trato urinário pelo tumor primário e sinais clínicos decorrentes destas obstruções (18, 19).

2.2 Epidemiologia do CCT da bexiga

Dentre os diversos fatores relatados como predisponentes ao desenvolvimento de tumores de bexiga em cães, podemos citar inicialmente o componente genético hereditário como as variantes mutacionais; e exposição a agentes ambientais exemplificados por produtos químicos (11, 20-23). Glickman et al., 2004 (23) realizaram estudo com 83 cães da raça Scottish Terrier com TCC da bexiga urinária e 83 cães da mesma raça como controle para avaliar o efeito da exposição a herbicidas de grama ou jardim no risco de desenvolvimento deste tumor. Como resultado, a exposição a grama ou jardim tratados com phenoxy herbicida foi associada a maior risco de desenvolvimento de TCC. Em estudo semelhante em cães da mesma raça, porém, o uso de pesticidas tópicos anti pulga e carrapato não foi associado ao maior risco de desenvolvimento da doença (20).

Além do efeito relatado para alguns produtos químicos, a dieta também pode influenciar o desenvolvimento do TCC da bexiga (24). O consumo de qualquer vegetal mais de 3 vezes por semana foi associado a uma redução de 70% do risco de desenvolvimento deste tumor em cães da raça Scottish Terrier, assim como o consumo específico de vegetais yellow-orange e de folhas verdes foi associado a uma redução ainda maior (70 a 90%) deste risco (24).

Além desses fatores, o sexo e raça são altamente correlacionadas com a incidência de CCT (11, 21, 25, 26). Fulkerson e Knapp, 2015 (21) relatam um risco 21 vezes maior de desenvolver a doença para scottish terriers e 3 a 6,5 vezes maior para cães esquimós, shetland sheepdogs, west highland white terriers, keeshonds, samoyeds e beagles, quando comparados a cães sem raça definida. E os mesmos autores citam que de acordo com os registros do Banco de Dados Médicos Veterinários de cães com carcinoma de células transicionais (11), entre as raças menos afetadas, algumas aparecem com risco aumentado, como collies, fox Terriers, cães esquimós americanos, airedale terriers, chesapeake bay retrievers e schipperkes. E em diferentes trabalhos, algumas raças se repetem, como de Brot et al. (2018) (27) que encontraram valor significativo de maior prevalência respectivamente em 3 raças: scottish Terrier, shetlhand sheepdogs e west highland white terrier.

No que diz respeito à predisposição por sexo, as fêmeas são mais afetadas do que os machos na proporção relatada de 1,71: 1 a 1,95: 1, ou seja, as cadelas têm quase o dobro do risco de desenvolver CCT de bexiga (11, 22, 26, 27). Porém, entre variáveis que representam maior predisposição para a ocorrência da doença, o sexo é mostrado como um fator de risco menos pronunciado (11). Diferentemente, em humanos, os

homens são mais afetados do que as mulheres, e esse fator pode ser devido às diferenças ocupacionais no tipo de trabalho, bem como aos diferentes níveis de exposição a agentes carcinogênicos entre ambos os sexos (28, 29).

Embora animais de diferentes idades possam desenvolver um tumor na bexiga, o CCT ocorre frequentemente em cães com idade avançada, entre 9 e 11 anos de idade (5, 26, 27). Além disso, cães castrados têm maior probabilidade de serem afetados quando comparados a cães intactos de ambos os sexos (11, 25, 30).

2.3 Tratamento clínico convencional e prognóstico

O tratamento quimioterápico, isolado ou em associação, é muito utilizado para tumores vesicais em cães, e embora não seja curativo, pode promover o controle ou remissão da doença (26). Alguns dos fármacos explorados são mitoxantrona, carboplatina, vimblastina, cisplatina, gencitabina e doxorrubicina, sendo a mitoxantrona um quimioterápico de destaque no tratamento clínico (21, 31-33). Os inibidores COX 2 também são muito estudados e considerados uma boa opção para o tratamento clínico, especialmente em associação (21). Em um estudo (31) encontraram uma maior sobrevida no tratamento com mitoxantrona associada a piroxicam quando comparado ao tratamento com qualquer um destes dois fármacos isoladamente. Alguns pesquisadores (3) associaram piroxicam à mitoxantrona e à carboplatina em grupos separados, não encontrando diferença entre eles quanto ao período livre de doença.

Além dos tratamentos convencionais clinico e cirúrgico, a radioterapia vem sendo estudada como uma opção. Porém, seu uso é limitado e podem ocorrer complicações como cistitie e colite (21, 34). O fato da bexiga não ser um órgão fixo no

abdome também dificulta o procedimento, embora o uso de novos equipamentos e técnicas tenham facilitado este tratamento (35, 36). Em estudo de 2004, o uso da radiação não resultou em melhores respostas do que as encontradas com mitoxantrona e piroxicam (37); embora outro artigo de 2012 tenha obtido bons resultados com a associação de quimioterapia e radioterapia em 4 cães com CCT músculo invasivo (33).

Assim como a doença em humanos, o CCT em cães tende a ter um prognóstico ruim. A sobrevida total relatada é de 4 a 9 meses, com variações entre autores de acordo com o tratamento estabelecido (2, 3, 21, 27). Grande morbi-mortalidade também está associada a este tumor em humanos e o cão tem sido recentemente estudado como um modelo natural para o CCT músculo invasivo em humanos, devido às características semelhantes entre as duas espécies (27, 31, 38-41).

2.4 Terapias emergentes

O estudo oncológico comparativo entre cães e humanos tem permitido o conhecimento mais aprofundado sobre o CCT em cães, com surgimento de terapias emergentes (21, 42-44). Knollman et al. (2015) (45) revisaram o potencial de diversas terapias para o carcinoma urotelial humano, sendo as principais: inibidores tirosina quinase, inibidores de fatores de crescimento fibroblástico, inbidores de fatores de crescimento epidermal, agentes anti-angiogênicos, inbidores do ciclo celular e agentes imunoterapêuticos, como é o caso dos inibidores de *checkpoints*.

Outro agente terapêutico que tem se destacado nos últimos anos é o *Viscum album (VA)*, ou Mistletoe, cujo efeito antitumoral foi investigado em poucos artigos médicos de tumor de bexiga (46-49), mas não há relato de seu uso em cães com este tipo tumoral. Embora os resultados tenham sido controversos, Urech et al. (2006) (49)

demostraram um efeito antiproliferativo do *Viscum album* no tumor de bexiga *in vitro* para humanos, evidenciando seu potencial efeito terapêutico e a necessidade de mais estudos.

Além da pesquisa de novos marcadores visando alternativas eficazes, ressalta-se a importância do estudo pré-clínico para o estabelecimento dos novos tratamentos, onde as culturas de células nos permitem a investigação do efeito citotóxico dos potenciais novos medicamentos em tipos tumorais específicos (38, 43, 50). Seguindo a linha de estudos *in vitro*, Rathore e Cekanova, (2014) (38) caracterizaram quatro linhagens celulares de CCT canino para estudo da doença e Sakai et al. (2018) (43) utilizaram cinco novas linhagens celulares de CCT de cães para avaliação do efeito de um inibidor tirosina kinase, demonstrando ser a cultura celular uma importante ferramenta para o estudo pré-clínico deste tumor.

No que se refere a terapia local recentemente estudada, podemos citar a eletroquimioterapia (51). Um grupo de pesquisa avaliou seu efeito em 3 cães com carcinoma urotelial, associada à cistotomia, observando resultado positivo de remissão tumoral em 2 dos 3 casos, sendo que o 1º porém apresentou uroperitoneo por ruptura vesical evoluindo a óbito, o 2º desenvolveu insuficiência renal aguda, e o 3º caso mostrou bons resultados até o momento da publicação, indicando uso possível da técnica, porém cauteloso (51).

Considerando os artigos citados acima, nos é clara a importância da maior investigação sobre o comportamento biológico do CCT canino e sobre novos *targets* moleculares que possam ser alvo de novas terapias complementares, assim como já ocorre de modo consistente na doença humana.

2.5 Marcadores imuno-histoquímicos

Diversas proteínas vem sendo estudadas há anos no CCT humano, e algumas já também no CCT canino, onde sua presença e expressão são frequentemente associadas a parâmetros clínico-patológicos tumorais, assim como ao prognóstico da doença e mesmo ao diagnóstico em si; e uma das técnicas laboratoriais frequentemente utilizada na análise da presença e expressão de determinadas proteínas, seja esta avaliação semi-quantitativa ou quantitativa, é a imuno-histoquímica (21, 52-56).

Como exemplos de marcadores imuno-histoquímicos já investigados em cães quanto ao seu papel no diagnóstico, tratamento ou prognóstico podemos citar: uroplaquinas, Ki67, Cox-2, EGFR, HER-2 e survivina (41, 52, 54-59).

Tanto em humanos quanto em cães, a expressão das uroplaquinas II e III tem finalidade diagnóstica na maioria das vezes, indicando a origem urotelial do tumor e distinguindo quando necessário os CCTs mais diferenciados dos pouco diferenciados (60-62). O fator de crescimento epidermal (EGFR), fator de crescimento epidermal 2 (HER-2) e survivina, embora menos investigados quando comparado as uroplaquinas , também apresentaram importância diagnóstica em casos de CCT da bexiga de cães em estudos anteriores (57-59). Tanto o EGFR quando o HER-2 se demonstraram superexpressos nas células tumorais quando comparados as células normais, podendo ser utilizados como marcadores de malignidade (57, 58). E Rankin et al., 2018 (59) observaram que a expressão nuclear de survivina pode estar presente no TCC da bexiga canino, enquanto é ausente nas células normais. Além disso, o HER-2 é uma possível molécula alvo para novas terapias (57).

Ainda no que se refere a diagnóstico, a presença ou ausência de expressão pelas células tumorais de marcadores específicos permite a subdivisão diagnóstica do CCT de

bexiga humano em diferentes subtipos moleculares (55, 63, 64). Esta subclassificação não é utilizada na medicina veterinária.

O ki67, por sua vez, é um marcador de proliferação celular muito estudado em diversos tipos tumorais, cujos valores quantitativos vêm sendo também correlacionados a variáveis histológicas do tumor de bexiga, como o grau histológico, e também à sobrevida dos pacientes (53, 56, 65). E relacionada ao tratamento, a expressão de COX-2 indica possível susceptibilidade das células tumorais aos efeitos antineoplásicos dos anti-inflamatórios inibidores de COX-2 (66).

Além dos marcadores já investigados em medicina veterinária, existem aqueles cujo papel é completamente desconhecido em cães, como é o caso do GATA-3 e da Caveolina-1 (41, 52, 54, 67). O GATA-3 é um marcador de diferenciação celular urotelial (68), e a Caveolina-1 é uma proteína da família das proteínas integradoras da membrana, além de participar de processos como endocitose, transporte vesicular e de vias de sinalização, podendo estar relacionada à carcinogênese (69, 70). Embora o papel de ambos os marcadores ainda seja pouco elucidado no comportamento tumoral, ambos já foram correlacionados a fatores patológicos como variante e grau histológico no carcinoma urotelial humano (52, 54), enquanto nenhum estudo similar com os mesmos marcadores foi ainda realizado para o carcinoma urotelial canino.

3. OBJETIVO GERAL

Esta pesquisa teve por objetivo realizar um estudo clínico, morfológico e imuno-histoquímico em CTT caninos.

3.1 Objetivos específicos

- Realizar estudo retrospectivo epidemiológico e patológico de casos de CCT canino;
- Investigar o padrão proliferativo do CCT canino através da avaliação imuno-histoquímica do marcador Ki67, comparando os valores obtidos com demais variáveis patológicas;
- Realizar estudo imuno-histoquímico dos marcadores GATA-3 e Caveolina-1, já anteriormente estudados no CCT humano, a fim de investigar o significado de suas expressões e suas relações com demais variáveis no CCT de bexiga canino;

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Capítulo 2 - TRABALHO CIENTÍFICO 1(normas da revista)

Trabalho já enviado para a revista "*Open Veterinary Journal*", exceto pelas imagens e tabelas, que foram inseridas dentro do texto, para uma melhor compreensão.
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Article

Retrospective evaluation of clinical and morphological findings of canine bladder urothelial carcinomas

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Abstract

Background: Urothelial carcinoma (UC), also known as transitional cell carcinoma, is the most common canine bladder malignant tumor and represents a model for studying human bladder cancer. However, there are few data in the literature regarding the clinic-pathological characteristics of these tumors and their prognostic value. **Aim:** the aim of this study was to evaluate such factors, correlating them with the follow-up, in a group of 32 dogs with bladder UC. **Methods:** clinical data of these cases, submitted to São Paulo State University UNESP) and VetPat Private Laboratory (São Paulo/Brazil), were recorded between January 2000 and November 2019. For each case, formalin-fixed and paraffin-embedded sections were hematoxylin-eosin stained and histologically evaluated. Survival analysis were performed and prognostic value was found for the presence of lymphatic invasion and for treatment used. **Results:** dogs that had neoplastic lymphatic vessel invasion experienced lower overall survival compared to those without lymphatic invasion; and dogs that received vinblastine associated with surgery had higher global survival when compared to animals that received carboplatin associated with surgery. **Conclusions:** the results obtained are encouraging showing the importance of further studies regarding the prognostic value of the two factors demonstrated as potential survival predictors, especially the lymphatic vessel invasion.

Keywords: Dog, transitional cell carcinoma; prognostic factor; bladder tumor

1.Introduction

Urothelial carcinoma (UC) of the bladder represents 1 up to 2% of all tumors in dogs (Hayes, 1976; Norris et al., 1992; Knapp et al., 2000a), being the most common tumor subtype among all bladder neoplasms (Valli et al., 1995; Knapp et al., 2014). Considering the similarities between human and canine bladder urothelial carcinomas, dogs have been proposed as a model for human bladder UC (Knapp et al., 2014; De Brot et al., 2018; Elbadawy et al., 2019). In humans, however, only invasive bladder cancer has clinical relevance since non-invasive bladder cancer can be successful removed by surgery. Thus, dogs can be useful for the study of the invasive type of this tumor (Elbadawy et al., 2019).

Dogs of different ages can develop bladder urothelial carcinoma, but it commonly affects dogs between 9 and 11 years old (Elbadawy et al. 2019). Also, neutered dogs are more likely to be affected than intact ones (Knapp et al., 2000a; Bryan et al., 2007; Knapp et al., 2014), and females tend to be more affected than males (Knapp et al., 2000a; Griffin et al., 2018). Among the predisposing factors, genetic factors and environmental exposure to chemicals have been reported (Raghavan et al., 2004; Knapp et al., 2014; Fulkerson and Knapp 2015; Griffin et al., 2018).

The UC can be classified according to their location, histological type, differentiation grade, and local invasion (Valli et al., 1995; Fulkerson and Knapp 2015; Marvel et al., 2017). In the bladder, one of the most common locations of tumor development is the bladder trigone. In several cases, the neoplasm already presents some degree of infiltration into the wall bladder at the time of diagnosis (Fulkerson and Knapp 2015; Marvel et al., 2017). Previously, the degree of tumor infiltration was evaluated in 33 bladder urothelial carcinoma cases, and submucosal, muscular, and

transmural infiltration were observed in 45%, 36% and 12% of cases, respectively (Marvel et al., 2017).

Because UCs tend to be locally invasive, the dogs can lead to partial or total obstruction of the lower urinary tract (Knapp et al., 2000b; Knapp et al., 2014). About 56% of the cases have urethral involvement, and 29% of the affected males have prostatic involvement. In addition, metastases in regional iliac and pelvic lymph nodes or distant metastases can be found in 16% of patients at the time of diagnosis and in 50% at necropsy (Knapp et al., 2000b; Knapp et al., 2014).

The treatment of bladder tumor in dogs is very challenging, mainly in patients with advanced disease, while surgery is not always an option for tumors located in the trigone region (Norris et al., 1992; Knapp et al., 2000a; Liptak et al., 2004; Saulnier-Troff et al., 2008; Molnár and Vajdovich 2012; Fulkerson and Knapp 2015; Marvel et al., 2017). Due to the importance of bladder UC in veterinary medicine and comparative studies, and also to the lack of prognostic markers for this tumor, we aimed to retrospectively study natural cases of UC in dogs, correlating clinical and pathological data with the follow-up.

2. Materials and Methods

2.1 Study design

This was a retrospective non-randomized study, including patients' samples from São Paulo State University and VetPat Laboratory, Brazil, between January 2000 and November 2019. The inclusion criteria were patients who underwent tissue biopsy or surgical procedure for histopathological diagnosis, availability of formalin-fixed and paraffin-embedded tissue samples, with histological diagnosis of bladder UC and

clinical information. Clinical data were obtained from each animal's record, including the following parameters: age, breed, gender, and treatment option. Additionally, clinical follow-up was carried out to assess survival time, when available, through the consultation of medical records and contact with the owner or responsible clinician. Survival time was considered from the day of definitive diagnosis by histopathology, until death.

2.2 Histological analysis

For each case, a 4 µm-thick section from the paraffin blocks were stained with hematoxylin-eosin. The histological subtype was reviewed by three evaluators in a blind manner (VMG, VG and RL-A), according to the criteria proposed by the World Health Organization (WHO) (Meuten et al., 2004), and the histological graduation was performed according to Valli et al., (1995). In addition, tumor infiltration in the different layers of the bladder, and the presence of lymphatic invasion were also recorded.

2.3 Statistical analysis

The clinical and pathological variables were tabulated, and the prevalence was calculated through the percentage of occurrence among the total cases. Animals that died as a consequence of surgery were considered untreated. Animals that underwent surgery and received or not chemotherapy were divided into: surgery + carboplatin, surgery + vinblastine, surgery + carboplatin + tyrosine kinase inhibitor (toceranib), and surgery only. The results were presented as mean and standard deviation. The available survival information was compared with clinical-pathological parameters using the Kaplan–Meier method; the log-rank test was used to compare the survival curves

statistically. The survival analysis was performed using GraphPad Prism v.5.0 (GraphPad Software Inc., La Jolla, CA, USA).

2.4 Ethics statement

This study was previously approved by the Institutional Ethics Committee on the use of Animal in Research from São Paulo State University- UNESP (Protocol 50/2020). All owners signed an institutional informed consent allowing the use of the patient's samples in research.

3. Results

3.1 Clinical data

Among the 32 dogs considered in the study, 71.87% were female and 28.13% were male. 47% were elderly animals, aging from 9 and 12 years old, followed by 31% over 13 years old, and a minority of 22% between 5 and 8 years old. Among the 14 affected breeds, Poodle (25.81%) was the most frequent, followed by Lhasa apso (9,67%), Daschund (6,45%), Labrador (6,45%), Beagle (6,45%), Pitbull (6,45%), Doberman pinscher (6,45%), and Maltese (6,45%). Those with only one case (3,23%) were English bulldog, Yorkshire, Shih Tzu, Scottish terrier, and Chow Chow. Mixed breed dogs represented 9,67% of the cases.

Information on treatment was obtained from 14 animals: 10 underwent surgery and received chemotherapy, while 4 received only surgical treatment. Of the total of 10 animals that received chemotherapy, the most used drug was vinblastine (50%) and carboplatin (40%), followed by carboplatin + tyrosine kinase inhibitor (toceranib)

(10%). The only animal that received toceranib also received carboplatin. Clinical information is reported in Table 1.

Table 1. Clinical information from the 32 urothelial carcinoma affected dogs.

Case	Breed	Age(years)	Sex	Treatment	Survival (days)
1	Labrador	11	Male	Surgery + carboplatin	90
2	Poodle	15	Female	NI	NI
3	Poodle	14	Female	Surgery + vimblastin	101
4	Daschund	5	Female	NI	NI
5	Beagle	10	Female	Surgery + carboplatin	130
6	Pitbull	7	Female	NI	NI
7	Lhasa apso	8	Male	Surgery + carboplatin	85
8	Lhasa apso	15	Female	NI	NI
9	Daschund	8	Female	NI	NI
10	Mixed breed	14	Female	NI	NI
11	NI	7	Female	NI	NI
12	Lhasa apso	13	Female	NI	NI
13	Beagle	12	Male	Surgery + vimblastin	145
14	Poodle	15	Female	NI	NI
15	Poodle	10	Female	NI	NI
16	English bulldog	11	Male	Surgery + carboplatin	60
17	Doberman pinscher	12	Female	NI	NI
18	Poodle	13	Female	NI	NI
19	Mixed breed	10	Male	Surgery + vimblastin	180
20	Maltese	10	Female	NI	NI
21	Yorkshire terrier	8	Female	NI	NI
22	Shih tzu	12	Female	NI	NI
23	Poodle	13	Female	Surgery + vimblastin	201
24	Maltes	15	Male	NI	NI
25	Labrador	7	Female	NI	NI
26	Chow chow	9	Male	Surgery	NI
27	Doberman pinscher	9	Female	NI	NI
28	Scottish terrier	10	Male	Surgery	1
29	Pitbull	13	Female	Surgery	4
30	Poodle	12	Male	Surgery + carboplatin + toceranib	485
31	Poodle	12	Female	Surgery	425
32	Mixed breed	12	Female	Surgery + vimblastin	412

NI = No information

3.2 Pathological data

Of all 32 tumors evaluated, the prevalent histological subtype was the papillary and infiltrating UC (53.13%) (Figure 1), followed by the non-papillary and infiltrating subtype (46.87%) (Figure 1). All tumors (N=32) had an invasive pattern. The predominant histological grade was grade II (71.88%), followed by grade III (28.12%) (Figure 2); and no grade I tumors were identified. In 23 out of 32 samples, all bladder layers were represented in the histological section, and of these, 47.83% (11/23) showed tumor invasion up to the muscle layer. Also, in 18 out of 32 cases (56.25%), lymphatic vessels invasion was detected, being striking and extensive in some samples (Figure 3) (Table 2).

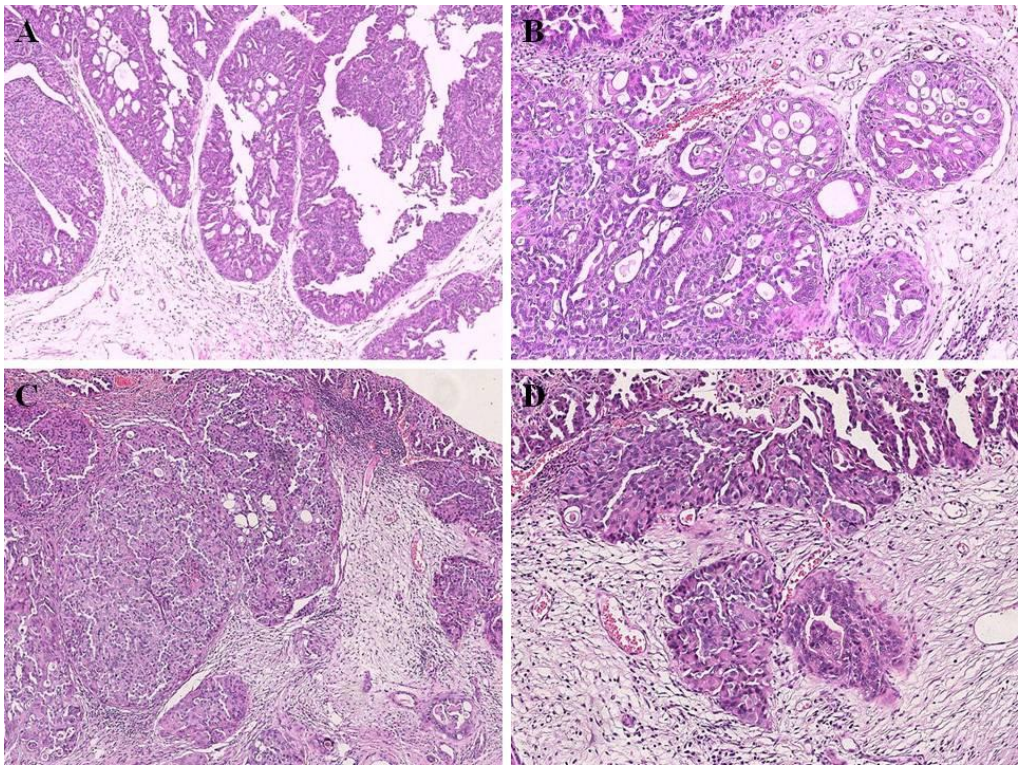

Figura 1. Histological type photomicrograph of canine UC. **A.** UC papillary (case 4). HE, 5x magnification. **B.** The same tumor of "A", focusing on tumor infiltration. HE, 10x magnification. **C.** UC non papillary and infiltrating (case 21). HE, 5x magnificatio

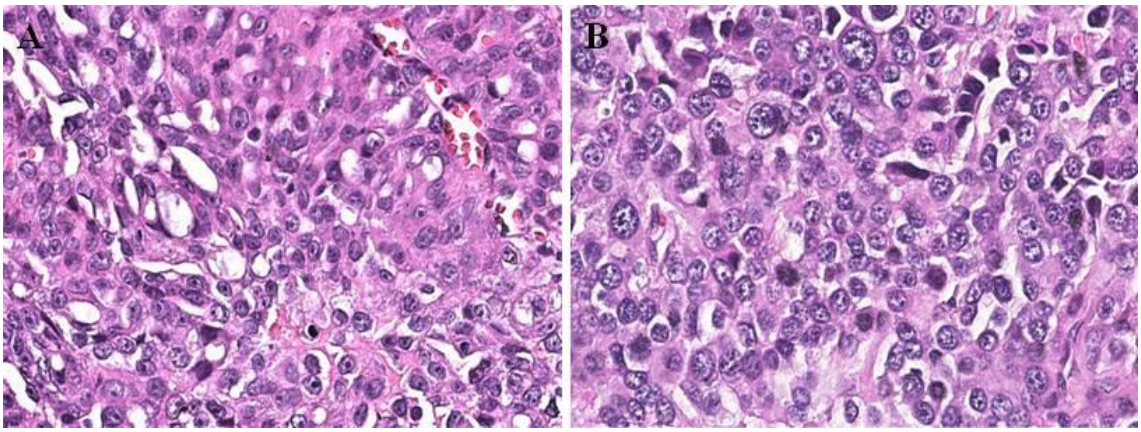

Figura 2. Histological grading photomicrograph of canine bladder UC. **A.** UC with histological grade II (case 11), HE, 40x magnification. **B.** UC with histological grade III (case 5), HE, 40x magnification. Enlarged nucleus, anisokariosis and multiple evident nucleoli

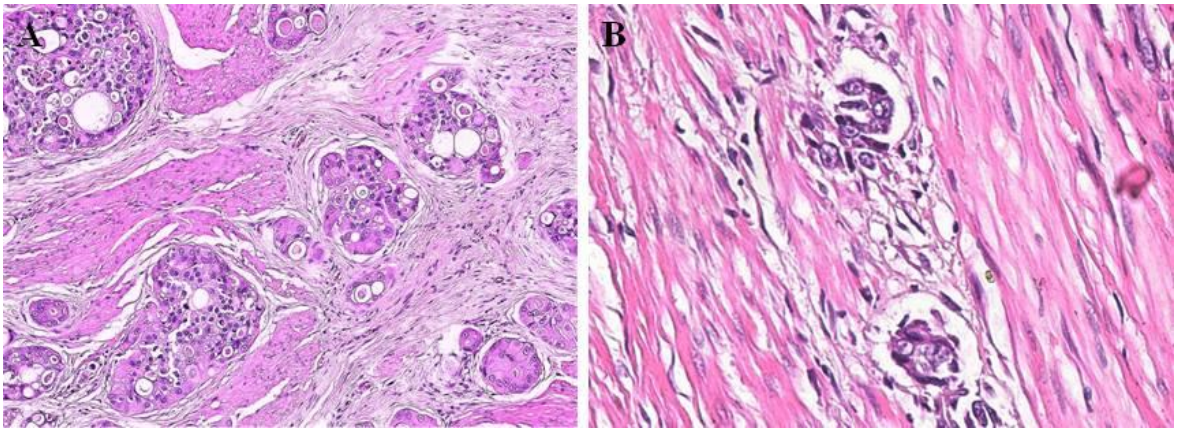

Figura 3. Histological photomicrograph of invasive canine bladder UC. **A.** UC with muscular infiltration (case 21), HE, 10x magnification. **B**. UC with lymphatic infiltration (case 12), HE, 40x magnification.

Table 2. Pathological data of the 32 canine bladder urothelial carcinoma cases.

Case	Histologic classification (WHO, 2004)	Histologic grade (Valli,1995	Sample with all layers	Muscular invasion	Lymphatic invasion
1	UC Papillary and infiltrating	II	Yes	Yes	present and extensive
2	UC Papillary and infiltrating	II	No	NI	absent
3	UC Papillary and infiltrating	II	No	NI	absent
4	UC Papillary and infiltrating	II	Yes	No	present
5	UC Non papillary and infiltrating	III	Yes	Yes	present and extensive
6	UC Papillary and infiltrating	II	No	NI	absent
7	UC Non papillary and infiltrating	III	Yes	Yes	present and extensive
8	UC Papillary and infiltrating	III	No	NI	present
9	UC Non papillary and infiltrating	II	Yes	No	absent
10	UC Non papillary and infiltrating	II	Yes	Yes	absent
11	UC Papillary and infiltrating	II	Yes	No	present
12	UC Non papillary and infiltrating	II	Yes	Yes	present and extensive
13	UC Papillary and infiltrating	II	Yes	No	absent
14	UC Papillary and infiltrating	II	No	NI	absent
15	UC Papillary and infiltrating	II	No	NI	present
16	UC Non papillary and infiltrating	II	Yes	Yes	present
17	UC Non papillary and infiltrating	II	Yes	No	present and extensive
18	UC Papillary and infiltrating	II	No	NI	present
19	UC Non papillary and infiltrating	III	Yes	Yes	present and extensive
20	UC Non papillary and infiltrating	III	No	NI	present
21	UC Non papillary and infiltrating	III	Yes	Yes	present and extensive
22	UC Non papillary and infiltrating	III	Yes	No	present
23	UC Papillary and infiltrating	II	Yes	No	absent
24	UC Non papillary and infiltrating	III	Yes	No	present
25	UC Papillary and infiltrating	II	Yes	No	absent
26	UC Papaillary and infiltrating	II	Yes	Yes	absent
27	UC Non papaillary and infiltrating	II	Yes	No	absent
28	UC Papaillary and infiltrating	II	Yes	No	present
29	UC Non papillary and infiltrating	III	Yes	Yes	present
30	UC Papaillary and infiltrating	II	Yes	No	absent
31	UC Papaillary and infiltrating	II	Yes	Yes	absent
32	UC Non papaillary and infiltrating	II	No	NI	absent

NI = No information

3.3 Survival analysis

Survival analysis were performed in association with the following parameters: type of treatment, histological subtype, histological grade, presence of muscle invasion and presence of lymphatic invasion. Of the 32 animals considered in the study, survival data (in days) were obtained for 13 cases. Of this total of 13 animals with bladder UC, 1 died due to post-surgical complications (4-day survival), and another one underwent euthanasia during surgery, due to the finding of an advanced stage disease, incompatible with less radical techniques of surgical tumor removal (1-day survival). The highest survival was 485 days for an animal that is still alive (treated with surgery + carboplatin + tyrosine kinase inhibitor), and the mean overall survival was 178.38 ± 161.05 days.

The histological subtype (papillary or non-papillary), histological grade and presence of muscle invasion did not present statistically significant correlation with survival time (Figure 4). On the other hand, the type of treatment influenced survival time (P=0.0497). Considering the treatment groups, the most representative were, respectively, those who received vinblastine and carboplatin as single adjuvant therapy. It was observed that dogs with bladder UC who received vinblastine as adjuvant treatment had a longer survival compared to dogs that received carboplatin. Of the 3 still alive animals, each one received a different protocol with different chemotherapeutic agents, having in common only the surgical procedure.

The presence of lymphatic invasion also had a prognostic value for survival: animals without tumor lymphatic invasion showed longer survival than those with lymphatic vessels invasion.

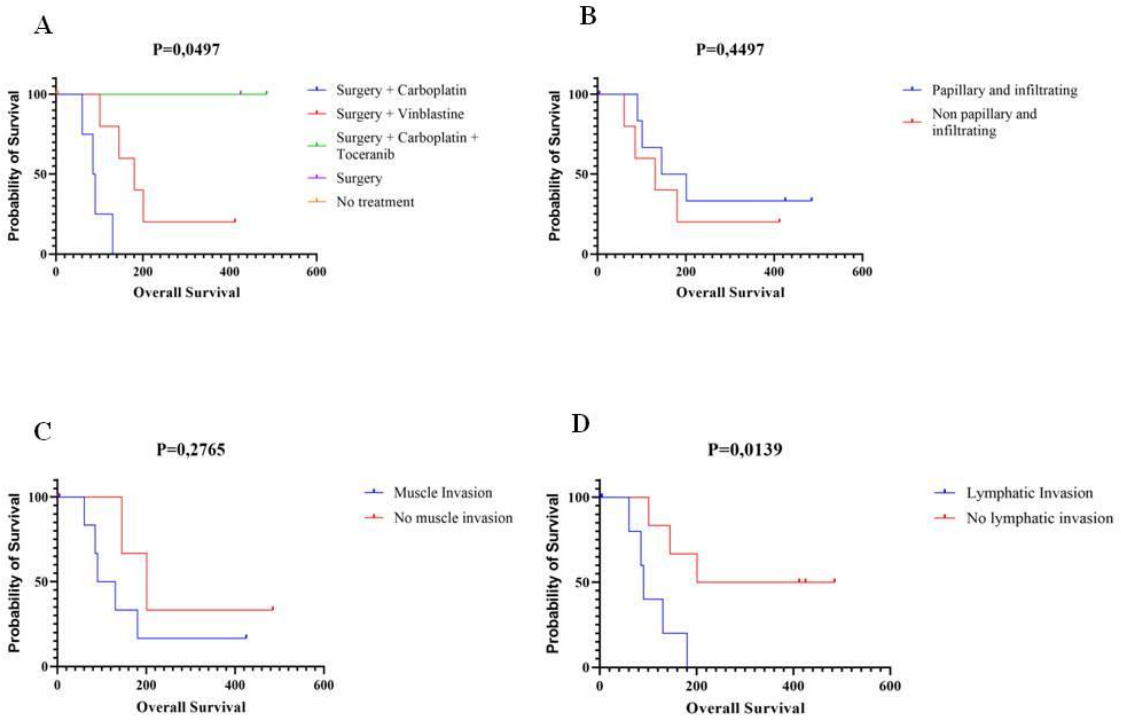

Figura 4. Survival curves (Kaplan-Meier) in relation to the different parameters. **A.** Survival curve for types of treatment. **B.** Survival curve for histological type. C. Survival curve for muscle invasion. D. Survival curve for lymphatic invasion.

4. Discussion

UC is the most common canine bladder tumor and represents a challenge in terms of treatment, due to its location and its frequent invasive behaviour. For its last features, the canine bladder UC has been proposed as a model for studying the muscle-invasive subtype of human bladder UC (Knapp et al., 2000a; Mutsaers et al., 2003). Considering the growing importance as a model for research in human medicine and the lack of information about this tumor in veterinary medicine, the present study aimed to describe the clinical-pathological features of canine bladder UC cases, investigating the prognostic value of some of them and emphasizing the importance of the epidemiological studies for greater knowledge about the behaviour of this tumor.

According to the literature (Knapp and McMillan, 2013; De Brot et al., 2018), most of the animals considered in the present study were females and elderly animals, between 9 and 12 years. The most commonly affected breed was Poodle, which is not one of the breeds mentioned as more prevalent in other countries. And among the total animals of this study, the breeds that are already reported by the highest probability of developing a bladder tumor (Fulkerson and Knapp, 2015) are Beagle and Scottish Terrier. This is probably due to differences in breed prevalence among the world's different regions and countries, deriving from cultural preferences and differences in the environmental condition such as the climate.

A recent meta-analysis has reviewed several studies related to bladder UC in both human and veterinary medicine, revealing that most studies of canine bladder UC do not assess pathologic features like tumor muscle invasion, and do not provide survival data, but considered only unique markers without investigating their prognostic value (Gambim et al., 2020). This fact reveals a low degree of standardization among veterinary practitioners and represents an important obstacle in searching for information in retrospective studies. Furthermore, the large number of biopsies collected by cystoscopies makes it impossible to assess the bladder's deeper layers to investigate the tumor invasion in most of the cases (Dhawan et al., 2015; Dhawan et al., 2018). Similar difficulties were found in the present study, where 13 out of 32 cases had information on survival time, and 23 out of 32 samples had all the layers sampled for histological evaluation of muscle invasion. In addition, the assessment of tumor invasion in dogs with UC is also not standardized. For this reason, in the present study the muscle layer and lymphatic vessels invasion by the tumor were both classified as present or absent.

In humans the muscle-invasive bladder UC represents only 30% of cases (Grzegółkowski et al., 2017). On the contrary, considering veterinary medicine, most bladder UCs show invasive behaviour, reaching the muscle layer, entering the lymphatic vessels or even affecting adjacent organs such as the urethra and prostate. This is probably due to the evolution of the disease at the time of diagnosis (Priester and McKay, 1980; Valli et al., 1995, Knapp et al., 2000a; Mutsaers et al., 2003). As confirmation of this invasive character, of the samples in the present study that allowed such assessments, 47.83% had muscle layer invasion and 56.25% had lymphatic invasion. The muscle invasion did not show a prognostic value using the Kaplan-Meier curve. On the other hand, the lymphatic invasion was evaluated in all samples and showed a prognostic value ($P = 0.0139$): animals with lymphatic vessels invasion had lower survival compared to those without lymphatic invasion. This result emphasizes the prognostic importance of considering this feature, which should always be evaluated and reported by the pathologist.

Other pathological characteristics, such as UC subtype and histological grade, did not show a significant difference in survival analysis, but we need to consider that the histological graduation described by Valli et al., (1995) evaluate only the nuclear features of tumor cells, introducing a possible bias in the application of such grading. For this reason, further studies comparing specifically the different grading system applied both in human and veterinary medicine are needed, to identify the one with the greatest prognostic value for de bladder UC.

Regarding the treatment of canine bladder UC, several conventional or emerging therapies have been used. Many of them have been transposed from human medicine due to the similarity between the diseases in the human and canine species (Knollman et al., 2015; Fulkerson et al., 2017). The dogs considered in this study were subdivided on

the base of the treatment received, to investigate the one that results in a longer survival time. Although the “surgery” and “Surgery + carboplatin + toceranib” treatment groups were represented by only 1 animal each, there was a statistically significant difference between the other types of therapy (P = 0.0497).

Allstadt et al., (2015) compared the use of piroxicam in association with carboplatin or mitoxantrone. Although they did not find a significant difference in patient survival, they observed a difference in the disease-free interval, where animals receiving carboplatin showed a shorter disease-free period (73.5 days) than those receiving mitoxantrone (106 days). Andold et al., (2011) conducted a clinical trial with vinblastine in dogs with bladder UC and observed a median survival of 147 days and a disease-free period of 122 days. Although the two studies mentioned above considered different animals and conditions, the disease-free period results were higher for the animals that received vinblastine than those that received carboplatin or mitoxantrone associated with piroxicam. In addition, a clinical trial by Chun et al., (1997) demonstrated that carboplatin was not beneficial in the treatment of 14 dogs with bladder UC. In the present study, dogs that received vinblastine as adjuvant therapy had a longer survival time than those who received carboplatin as adjuvant therapy. This shows us a better therapeutic potential of vinblastine compared to carboplatin. Still considering the treatment options, surgery is the first option and offers good results in terms of survival. However, as stated above, its execution is not always possible or compatible with the animals' quality of life (Molnár and Vajdovich, 2012). Patients with tumors located on the bladder trigon represent a challenge in performing a surgical procedure. On the contrary, when the tumor is not located on the bladder trigone, good results are achieved by partial cystectomy associated with clinical treatment (Marvel et

al., 2017). In this study's therapeutic groups, only one animal received surgery exclusively, making it difficult to make an assumption about this method alone.

Another predictor of prognosis, which also interferes with the treatment to be performed, is the tumor location within the bladder and other structures' involvement. Allstadt et al., (2015) noted that males with prostatic involvement had lower median survival. Again, animals with bladder trigone involvement had lower survival than animals with urethral involvement and animals with apical tumors. Those with tumors at the bladder apex had higher median survival (Allstadt et al., 2015). For the dogs considered in the present study, no information was obtained regarding the tumor's location, hampering to evaluate such a variable's prognostic importance.

This article evaluates the prognostic value of different treatment options and tumor histological features of canine bladder UC. In particular, lymphatic invasion, a feature scarcely considered in the available literature, proved to be a significant negative prognostic factor. The results reported above stimulate the continue study of canine bladder UC, not only for application in translational research with human disease, but also for developing new therapies that enable better prognosis and greater quality of life in veterinary medicine.

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V.M.G, C.E.F-A., V.G., R.L-A.; formal analysis, V.M.G, C.E.F-A.; investigation, V.M.G., C.P., F.A.R.S., C.E.F-A., R.L-A. V.G., T.O.daS., F.Z.; resources, C.E.F-A., R.L-A; data curation, V.M.G., C.P., C.E.F-A, V.G., R.L-A, T.O.daS., F.Z. ; writing—original draft preparation, V.M.G..; writing—review and editing, V.M.G., C.E.F-A., C.P., R.L-A., V.G.; visualization, V.M.G., C.E.F-A., R.L-A., C.P., V.G.; supervision, C.E.F-A.; project administration, C.E.F-A., J.G.Q., V.G. All authors have read and agreed to the published version of the manuscript.

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Capítulo 3 - Trabalho Científico 2

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Article

CAVEOLIN-1, GATA-3 AND KI67 EXPRESSIONS AND THEIR CORRELATION WITH PATHOLOGICAL FINDINGS IN CANINE BLADDER UROTHELIAL CARCINOMA

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Simple Summary: The bladder urothelial carcinoma (UC) represents a challenge in veterinary medicine and there are few informations about marker associated with the disease development. The present study aim to investigate the biomarkers Caveolin-1, GATA-3 and Ki67, , correlating those withpathological variables. As occour in human, we identified a strong GATA-3 expression in urothelial carcinomas and also identifed association of GATA-3 and Caveolin expression with limphatic invasion, the histophatological type and tumor grade. Thus, these markers could potentially be used to evaluate agressiveness of these tumors. Overall, our results indicated that Caveolin-1 and GATA-3 could be assessed on histological samples to preditic patients prognosis.

Abstract: The bladder urothelial carcinoma carcinoma (UC) represents about 2% of malignant neoplasms in dogs, and is a challenge in veterinary medicine in terms of treatment. There are few informations regarding catarcteristics of the bladder tumor in dogs, such as the presence of muscle invasion and the prognostic value of molecular markers. The present study aimed to study the Caveolin-1, GATA-3 and Ki67 mmunostaining in canine UC samples, evaluating their correlations with histopathological variables. Thirty paraffinized tumor samples were obtained and stained by HE staining and Caveolin-1, GATA-4 and Ki76 expression was assessed by immunohistochemistry and associated with pathological factors. When performed a univariate analysis, no association were found between each marker and the pahtological markers. Thus, we performed a multivariate analysis among all markers and pathological data and ideitfed a positive correlation between GATA-3 and mitotic (MC) and a negative correlation between Caveolin-1 and MC. Moreover, lymphatic invasion was positively correlated with histological the type and grande, and negatively correlated with MC. Besides that, hitological type was positiviley correlated with histoligcal grade. Overall, our results indicated that Caveolin-1 and GATA-3 expression could be promissing as markers of bladder urothelial carcinoma agressiveness,

Keywords: Cancer, dog, biomarker, immunohistochemistry

1.Introduction

Urothelial carcinoma (UC) represents 1.5 to 2% of naturally-occurring cancers in the canine species, and due to the similarities between this disease in dogs and humans, the dog's muscle-invasive bladder tumor has been used as a study model for the disease (1-4). Although invasive bladder UC is lethal in 50% of cases in humans (1), about 70% of patients have a non-invasive bladder tumor (5, 6), and in the other hand, in dogs the disease is characterized by the invasive form in most cases (4). In addition, the majority of canine UC is located in trigone and 50% already have urethral involvement, while in humans the location tends to be more variable (1, 4). Due these factors, the treatment of the dog's bladder tumor is a challenge in veterinary medicine.

Considering the difficulty faced by veterinary doctors in the treatment of canine bladder UC and the lack of knowledge about this disease when compared to the humans' disease, several studies have been conducted with several biomarkers in the search for new therapeutic targets and even more information about the biological behavior of this cancer (7, 8). Gambim et al. (7) demonstrate that most part of the studies in veterinary medicine are limited to a group of proteins, based on human studies. Thus, different manuscript usually evaluates the same protein (HER-2 and EGFR). These authors performed a in silico analysis and demonstrated the potential of Caveolin-1 and GATA-3 expression as markers for canine urothelial carcinomas (7). However, to the best of author's knowledge, no previous studies reported the expression of these markers on canine UC.

Caveolin-1 expression has also been investigated for human UC, both in the bladder and in the upper urinary tract, and correlated with clinicopathological factors and disease progression (9, 10). This is a protein involved in several cellular biological processes, such as endocytosis, vesicular transport and signaling pathways (11, 12). In veterinary medicine there are no studies investigating the predictive value, or the possible correlation between the expression of these two biomarkers and other clinic-pathological variables for canine bladder UC.

GATA binding protein 3 (GATA-3) is a zinc finger transcription factor mainly involved in the differentiation and cell specification processes of tissues such as urothelium and breast epithelium (13, 14). It has been studied as a marker for human urothelial carcinoma, both related to diagnosis and correlated to clinicopathological factors such as histological grade, histological type, and staging, in order to investigate its role as a predictor of the behavior of this tumor type (15-17).

The ki67 is an example of a biomarker widely used in both human and veterinary oncology to establish prognostic estimates, either alone or in association with other markers (6, 18). Protein ki67 expression acts as an indicator of cell proliferation within a population of cells (19), and this expression has already been evaluated for canine cancers like lymphoma, melanoma, mast cell tumor and mammary tumor (18, 20-22). With regard bladder UC, several articles in humans bring results that point to Ki67 as an important factor to be considered in the prognosis and tumor behavior, related to disease progression, histological grade or the stage of the disease (6, 23-27). And in veterinary medicine, in the best of our knowledge exist only one study that investigated the expression of Ki67 in the canine bladder UC with no statistically significant correlation between this marker and the animals' survival time (28), showing the importance of more studies related to Ki67 for this tumor type, also associating it with histopathological features.

Thus, this article aimed to evaluate Caveolin-1, GATA-3 and Ki67 expression in canine UC samples, correlating this with other histopathological variables.

2. Materials and Methods

2.1 Ethic statement

This study was previously approved by the Institutional Ethics Committee on the use of Animal in Research from São Paulo State University- UNESP (Protocol 50/2020). All owner signed an institutional informed consent allowing the use of the patient's samples in research.

2.2 Study design

This was a retrospective non-randomized study, including 30 patients' samples, being 21 from VetPat Laboratory and 9 from University of Milan between January 2000 up to June 2019. The inclusion criterion were patients that underwent tissue biopsy or surgical procedure for the acquisition of tissue sample, availability of tissue sample in paraffin block and clinical information. Clinical data were obtained from the records of each animal and the following histopathological parameters were evaluated: histological type and grade, muscle invasion, lymphatic invasion and mitotic index. Those samples whose material was insufficient to determine the histological type (infiltrating or not infiltrating) or which presented negative internal control for the markers, were excluded.

2.3 Histological analysis

A new tissue section was performed from the paraffin blocks and hematoxylin and eosin staining was performed for the tumor classification. The histological subtype were obtained by three evaluators (VMG, VG and RL-A) according to the criteria proposed by the World Health Organization (23) (29) and the histological graduation was performed according to Valli et al. (30). In addition, the presence or absence of tumor infiltration in the muscle layer of the bladder were reported; and the presence or absence of lymphatic invasion was also reported. Besides that, mitotic count was analyzed counting the total of mitoses present in fields with high mitotic activity totalizing an area of 2,37mm^2 (400x magnification), according to Romansik et al. (31).

2.4 Immunohistochemical analysis

2.4.1 ki67

From each formalin fixed and paraffin embedded samples, 5μm-thick sections were cut. Histological sections were deparaffinized and rehydrated through graded alcohols, and endogenous peroxidase activity was blocked for 30 min in a 0.3% H2O2 methanol solution. Heat-induced antigen retrieval was obtained with a pressure cooker for 20 min in citrate buffer pH 6.0. After washing in Tris-buffer, protein blocking was performed with horse normal serum for 30 min at 25 °C. Sections were then incubated for 18h at 4°C with a mouse anti-human Ki-67 primary antibody (MIB-1, Dako, CA, USA) diluted 1: 600 in Tris-buffer. After rinsing sections in Tris-buffer, slides were incubated for 30 min at 25°C with a biotinylated horse anti-mouse secondary antibody (Vector Laboratories, CA, USA). The immunohistochemical signal was detected using an avidin-biotin system (Vector Laboratories, CA, USA) and AEC (3-amino-9-ethylcarbazole) substrate - chromogen kit (Vector Laboratories, CA, USA). Sections were counterstained with Harris Hematoxylin and mounted with an aqueous mounting medium (Aquatex, Sigma-Aldrich, MO, EUA).

Ki-67 value was expressed as the percentage of positive-stained cells calculated counting a total of 1000 cells per section (400x magnification).

2.*4.2 GATA-3 and Caveolin-1*

Samples were processed in the same way as for the Ki67 marker. After deparaffinization, the sections were submitted to the antigenic retrieval procedure: Caveolin-1 - in citric acid solution (pH 6.0) in a pressure cooker; and GATA-3- in a high pH buffer solution (EnVision Flex, High pH, Dako, CA, USA) in water bath at 98°C, for 30 minutes. Endogenous peroxidase was blocked with 0.5% H2O2 methanol solution for 20 minutes; and later with diluted powdered milk (3g in 100ml) at 25°C for 1 hour. The slides were incubated in a humid chamber, overnight and at 4°C, with the primary antibodies: anti-human monoclonal mouse GATA-3 (L50-823, Biocare Medical, CA, USA), diluted 1:100; and CAV1 anti-human polyclonal (AVARP09019_T100, Aviva Systems Biology, CA, USA), diluted 1:1000. After washing with Tris-buffer, the tissues were incubated with secondary antibody (EnVision Flex SM802, Dako, CA, USA) at 37°C for 1 hour; and the immunohistochemical signal was detected using a diaminobenzidine solution (DAB Chomogen Kit, Dako, CA, USA). The counterstained were performed with Harris Hematoxylin.

The expression of caveolin-1 was analyzed semi-quantitatively by the criteria intensity of labeling from 1 to 3, and tumor staining distribution from 1 to 4 (1=1 to 25%, 2=26 to 50%, 3=51 to 75%, 4 =>75%). Intensity and distribution values were then multiplied, resulting in a value from 1 to 12 representative of the expression of this marker. The expression of GATA-3 was analyzed quantitatively, and its value was expressed as the percentage of positive-stained cells calculated counting a total of 1000 cells per section (400x magnification).

2.5 Statistical analysis

The pathological variables were tabulated, and the prevalence was calculated through the percentage of occurrence among the total cases. In this case, the results are presented as mean and standard deviation. The biomarkers values and the histopathological information's were associated in univariate analysis using t-student or Mann-Whitney test when the pathological variable was composed by two groups; or the Kruskal Wallis test when the variable was composed by three groups. And multivariate analysis was performed to correlate all variables using Spearman's correlation test. The correlation coefficients (r) were interpreted according to Pett, Lackey and Sullivan (32), dividing the classifications into weak (0<r>0,29), low (0,30<r>0,49), moderate (33), strong (9) or very strong (0,90<r>1); whether they are positive or negative. The weak correlation was not considered.

The muscular invasion variable was not considered in the tests because of the smaller number of cases with this information. The software used was GraphPad Prism v 8.0.1 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1 Clinicopathological and immunohistochemical data

Initially, 40 canine UC samples were selected for histopathological and immunohistochemical assessment for Caveolin-1, GATA-3 and Ki67. After excluding samples for which it was not possible to determine the histological type, and those with negative internal control for the biomarkers, there were a total of 30 samples used in the present study (Table 1).

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Table 1. Clinicopathological and immunohistochemical data

CASE	SEX	AGE (YEARS)	HISTOLOGIC CLASSIFICATION (WHO, 2004)	HISTOLOGIC GRADE (30)	SAMPLE WITH ALL LAYERS	MUSCULAR INVASION	LYNFHATIC INVASION	MITOTIC COUNT (MC)	Ki67	CAVEOLIN-1	GATA-3	SURVIVAL (DAYS)
1	Male	11	TCC Papillary and infiltrating	II	Yes	Yes	PRESENT AND EXTENSIVE	2	6,05%	(3x3)=9	51,70%	90
2	Female	15	TCC Papillary and infiltrating	II	No	NI	ABSENT	39	40,20%	(4x3)=12	35,75%	NI
3	Female	14	TCC Papillary and infiltrating	II	No	NI	ABSENT	4	25,09%	(2x3)=6	70,24%	101
4	Female	5	TCC Papillary and infiltrating	II	Yes	No	PRESENT	24	45,91%	(4x3)=12	91,64%	NI
5	Female	10	TCC Non papillary and infiltrating	III	Yes	Yes	PRESENT AND EXTENSIVE	27	30,80%	(2x1)=2	87,04%	130
6	Female	7	TCC Papillary and infiltrating	II	No	NI	ABSENT	28	42,72%	(1x1)=1	23,32%	NI
7	Female	15	TCC Papillary and infiltrating	III	No	NI	PRESENT	17	20,33%	(4x3)=12	66,94%	NI
8	Female	8	TCC Non papillary and infiltrating	II	Yes	No	ABSENT	120	76,34%	(2x2)=4	90,96%	NI
9	Female	14	TCC Non papillary and infiltrating	II	Yes	Yes	ABSENT	7	3,48%	(2x2)=4	88,18%	NI
10	Female	7	TCC Papillary and infiltrating	II	Yes	No	PRESENT	22	33,33%	(3x2)=6	70,28%	NI
11	Female	15	TCC Papillary and infiltrating	II	No	NI	ABSENT	81	27,98%	(1x2)=2	87,21%	NI
12	Female	10	TCC Papillary and infiltrating	II	No	NI	PRESENT	12	35,66%	(3x2)=6	75,28%	NI
13	Male	11	TCC Non papillary and infiltrating	II	Yes	Yes	PRESENT	7	34,40%	(4x3)=12	77,61%	60
14	Female	12	TCC Non papillary and infiltrating	II	Yes	No	PRESENT AND EXTENSIVE	78	54,97%	(1x2)=2	87,37%	NI
15	Female	13	TCC Papillary and infiltrating	II	No	NI	PRESENT	31	10,33%	(1x2)=2	80,51%	NI
16	Male	10	TCC Non papillary and infiltrating	III	Yes	Yes	PRESENT AND EXTENSIVE	66	14,25%	(2x2)=4	71,04%	180
17	Female	10	TCC Non papillary and infiltrating	III	No	NI	PRESENT	51	16,27%	(1x2)=2	88,30%	NI
18	Female	8	TCC Non papillary and infiltrating	III	Yes	Yes	PRESENT AND EXTENSIVE	12	33,38%	(3x2)=6	89,04%	NI
19	Female	12	TCC Non papillary and infiltrating	III	Yes	No	PRESENT	21	24,48%	(4x3)=12	66,86%	NI
20	Female	13	TCC Papillary and infiltrating	II	Yes	No	ABSENT	22	29,53%	(1x2)=2	58,42%	201
21	Male	15	TCC Non papillary and infiltrating	III	Yes	No	PRESENT	22	11,69%	(3x2)=6	70,35%	NI
22	Male	10	TCC Non papillary and infiltrating	III	Yes	Yes	ABSENT	28	25,36%	(3x2)=6	61,94%	NI
23	Female	13	TCC Papillary and infiltrating	II	Yes	No	ABSENT	30	6,84%	(2x1)=2	78,09%	NI
24	Male	11	TCC Papillary and infiltrating	II	Yes	Yes	PRESENT	17	10,19%	(1x1)=1	78,90%	NI
25	Male	8	TCC Papillary and infiltrating	II	Yes	Yes	ABSENT	NI	8,24%	(4x3)=12	78,88%	NI
26	Female	12	TCC Papillary and infiltrating	II	No	NI	ABSENT	32	17,72%	(3x3)=9	84,88%	NI
27	Female	15	TCC Papillary and infiltrating	II	Yes	No	ABSENT	28	4,78%	(4x3)=12	85,14%	NI
28	Male	NI	TCC Papillary and infiltrating	I	No	NI	ABSENT	NI	4,31%	(2x3)=6	56,13%	NI
29	Male	15	TCC Non papillary and infiltrating	III	Yes	Yes	PRESENT	27	12,75%	(3x3)=9	79,43%	NI
30	Male	10	TCC Papillary and infiltrating	I	Yes	No	ABSENT	36	16,84%	(3x2)=6	89,55%	NI

NI = no information

All samples were classified for the histological type, divided into papillary and infiltrating (60%) and non-papillary and infiltrating (40%), with the invasive characteristic being observed in all cases, and the papillary component in most of them. The histological grade II by Valle et al., 1995 was observed in 63.33% of the cases, followed by grade III (30,01%) and grade I (6.66%) (Table 1).

Of the total of 30 bladder tumors, 20 presented all layers sampled for histological evaluation of muscle invasion. Of these complete samples, 50% already had some infiltration of the bladder muscle layer. In addition, for the 30 tumor samples, all were evaluated for lymphatic invasion, and 53.33% of them demonstrated the presence of invasion of lymphatic vessels (Table 1).

For histological evaluation of the cell proliferation of the canine UC, the MC was considered. A total of 28 samples allowed the determination of MC through the visualization of the necessary tumoral area (Table 1), and the MC mean ± standard deviation was 31,82 ± 26,26.

As a result of the immunohisdifferentel analysis of ki67, GATA-3 and Caveolin-1, the median ± standard deviation of immunostaining values were respectively, 24,14 ± 16,88%, 71,40 ± 16,38 % and 6,23 ± 3,95. (Table 1) (Figure 1). The quantitative expression of GATA-2 was higher than 75% in 60% of the cases, and the semiquantitative expression value of Caveolin-1 was ≥ 6 in also 60% of the canine UC cases. Examples of differents expressions of Ki67, GATA-3 and Caveolin-1 are illustrated in Figure 1.

Survival data were retrieved from only 6 of the total dogs, and the median overall survival was 127 days from the diagnosis.

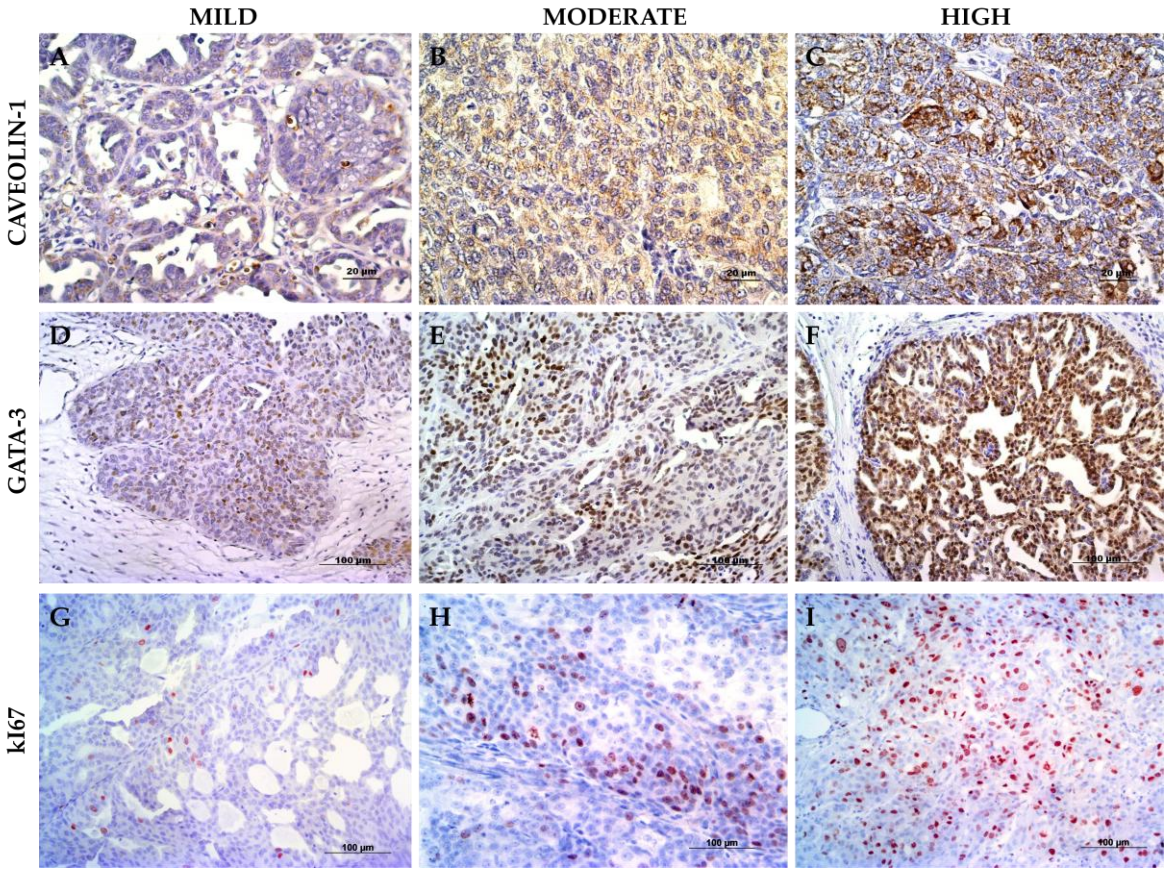

Figure 1. Immunostaining panel. **A.** UC papillary and infiltrating with mild Caveolin-1 (case 6), 400x magnification. **B.** UC papillary and infiltrating with intermediate Caveolin-1 (case 30), 400x magnification. **C.** UC papillary and infiltrating wit high Caveolin-1 (case 7), 400x magnification. **D.** UC papillary and infiltrating with mild GATA-3 (case 2), 200x magnification. **E.** UC non papillary and infiltrating with intermediate GATA-3 (case 22), 200x magnification. **F.** UC papillary and infiltrating wit high GATA-3 (case 30), 200x magnification. **G.** UC papillary and infiltrating with mild ki67 (case 15), 200x magnification. **H.** UC papillary and infiltrating with intermediate Ki67 (case 26), 200x magnification. **I.** UC non papillary and infiltrating with high Ki67 (case 14), 200x magnification.

3.2 Associations between all pathological and immunohistochemical variables

For the study of the association between the variables, the 30 samples were considered, except for mitotic count, for which the information could be determined only for 28 tumors. No statistically significant association was found for any histopathological or immunohistochemical variable in the univariate analysis (Table 2).

Table 2. P values for the univariate analysis. MC and immunohistochemical markers values were associate with each other and with the histopathological variables, without statistically significant results.

X	**Mitotic Count**	**Ki67**	**Caveolin-1**	**Gata-3**
Histological type	0,7401	0,4648	0,6616	0,1461
Histological grade	0,6489	0,3742	0,8892	0,9916
Linphatic invasion	0,1235	0,5249	0,7857	0,6084
Mitotic count	----	0,3466	0,099	0,0917
Ki67	0,3466	----	0,8484	0,6595
Caveolin-1	0,099	0,8484	----	0,4415
Gata-3	0,0917	0,6595	0,4415	----

In the multivariate analysis of multiple correlations, some interesting results were found. (Figure 4). With regard to immunohistochemical markers, GATA-3 expression was positively correlated with MC (low correlation, r=0.32), that is, higher GATA-3 values tended to correspond to higher MC values. And Caveolin-1 was negatively correlated with MC (low correlation, r= -0.32), showing that higher values of these markers tended to correspond to lower MC values. The ki67 was not significantly correlated to any variable (Figure 4).

With regard to the histopathological variables, lymphatic invasion showed a low positive correlation with the histological type (r= 0.31) and with the histological grade (r= 0.47), and a low negative correlation with the MC (r= - 0.30). These results indicate that the non-papillary histological type as well as higher histological grades tended to show lymphatic invasion, while tumors with higher MC tended not to show lymphatic vessel invasion. In addition, histological grade and type were positively correlated each other with a moderate correlation coefficient (r=0.64), indicating that non-papillary histological type tumors tended to have a more advanced histological grade (Figure 4).

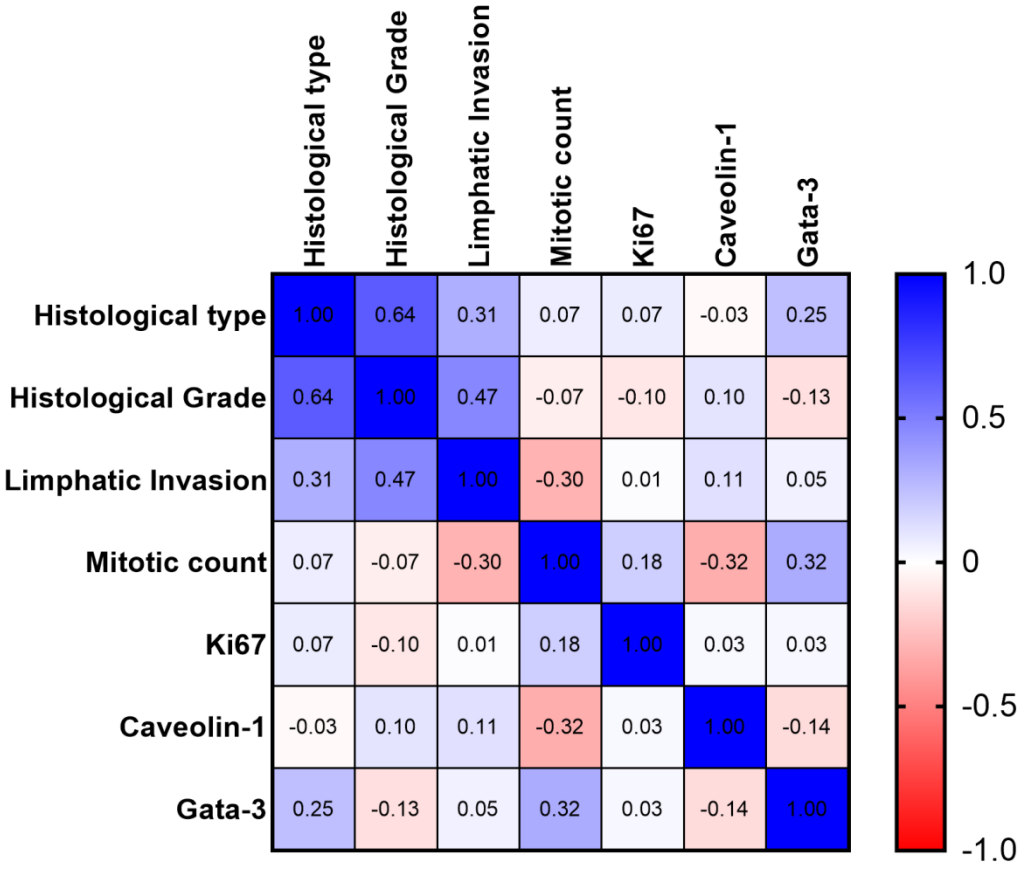

Figure 2. Spearman's graph of multiple correlations with the respective correlation coefficients. The blue color indicated a positive correlation and the red color a negative correlation. The intensity of the color is related with a stronger correlation (34). It is observed a strong correlation between histological grade and histological type and a moderate negative correlation between mitotic count and Caveolin-1 expression.

4. Discussion

The canine bladder UC is known for its increased invasive character at the time of diagnosis, being an important model for muscle-invasive disease in humans (1, 4). However, despite the difficulties associated with the treatment of this tumor type in canine species, most studies related to biomarkers for bladder UC in veterinary medicine, analyze isolated molecules without assessing their prognostic value or their association with other variables (7) . And the same study performed a systematic review and meta-analysis evaluating genes and proteins in human and canine bladder tumor, pointing proteins with prognostic predictive value in the human tumor that are not studied in the veterinary medicine yet. Based on these facts, the present study aims to study the expression of Ki67, GATA-3 and Caveolin-1and their meanings in relation to the main histopathological variables for the canine bladder UC.

Of the 30 cases studied, all were classified histologically as infiltrating, with 50% of 20 samples showing muscle invasion, and 53.33% of the total showing invasion of lymphatic vessels. These results reinforce the invasive characteristic of most cases of bladder tumor in dogs and the importance of studying new predictive biomarkers.

The role of Ki67 in tumor behavior has been extensively studied in several cancers in veterinary medicine, such as mast cell tumor, for example (18). Similarly, for human TCC, several studies have evaluated the prognostic value of Ki67. Chirife et al. (24) reported ki67 as a predictor of bladder tumor progression in patients undergoing initial transurethral resection; and meta-analysis of He et al. (6) found a statistically significant result between overexpression of ki67 and a shorter progression free survival. The present study did not correlate de ki67 values with survival data and because of the small number of cases with overall survival reported, and also the retrospective character do not allow the evaluation of variables such as progression free survival.

The association of Ki67 values with other histological variables is also important for a better understanding of the tumor biology of bladder UC, both in humans and in dogs. Gönül et al. (23) observed an association between this biomarker and the tumor grade in humans; however, the same association was not found for the cases in the present study. And although in human medicine there are many articles investigating the predictive value of Ki67 for bladder UC, in the best of our knowledge, Hanazono et al. (28) were de first authors to investigate the Ki67 value correlating that with survival time, being the present study the second one to correlate ki67 with other two biomarkers and histological features. Therefore, the fact that no significant statistical correlation was found between ki67 and any other variable, stimulate the execution of more studies, with a bigger number of animals and with more follow-up information.

As well as Ki67, other markers have been studied in terms of their significance in tumor behavior for human UC, such as GATA-3 and Caveolin-1 (9, 10, 15-17), and on the other hand, there are no veterinary articles that investigated these markers for the same disease in dogs, with the exception of a comparative study that uses the dog as a model of human disease, subclassifying canine bladder carcinomas into molecular subtypes (11). Always for human disease yet, Naik et al. (16) observed a lower positivity of this marker in high-grade UC when compared to low-grade UC, also in tumors with muscle invasion when compared to those with invasion only in lamina propria, and in non-papillary tumors when compared to papillary histological types. Leivo et al. (35) did not demonstrate prognostic significance of GATA-3 for UC; and Miyamoto et al. (15) pointed out that while this biomarker was less expressed for low-grade tumors and muscle-

invasive tumors, its strong immunostaining proved to be an independent predictor of poor prognosis. These results highlight the still controversial and poorly understood biological role of GATA-3 in UC.

In contrast to most of the correlations mentioned above for the disease in humans, the present study found a correlation of GATA-3 only with MC, which was positive and low, indicating that tumors with higher expression of GATA-e tend to have higher MC. Considering that high mitotic activity may be a predictor of a greater chance of tumor recurrence for human UC (36), and consequently more aggressive tumor behavior, this result suggests that higher GATA-3 positivity can indicate a worse prognosis for bladder cancer in dogs. However, it should be taken into account that all samples used already had an invasive character in lamina propria or muscle layer, making this sample set homogeneous for this item, which possibly influenced that the cases have tendentially higher mitotic index. The invasive character of the tumor samples may also have influenced the non-correlation of ki67 values with the other variables

Similar to GATA-3, Caveolin-1 expression was correlated in this study with MC. However, its correlation was low and negative, indicating that higher expression values of this marker tend to accompany lower MC. values The results found in humans are still unclear; Fong et al., 2003 (9) reported a positive correlation between caveolin-1 expression and tumor histological grade, and D'Andrea et al. (10) pointed to a positive association not only between caveolin-1 values and tumor grade, but also with more advanced stages of the disease. In contrast, this last study also showed that higher values of caveolin-1 were associated with lower disease recurrence. The results of the present study are contrary to most results in humans, as they indicate that high expression values of caveolin-1 can indicate a favorable tumor biological behavior, as they were inversely correlated with MC. Although that result, the same bias of the high histological invasiveness of tumor samples must be considered. Furthermore, both for GATA-3 and for Caveolin-1, the correlations found were classified as low, evidencing the need for further studies with the same biomarkers.

With regard to histological variables such as MC, histological type, histological grade and lymphatic invasion, low positive correlation was found between lymphatic invasion and histological type, and also between lymphatic invasion and the histological grade of Valli (1995), suggesting that non-papillary tumors as well as high histological grade tumors are more likely to present lymphatic vessel invasion. Considering that the presence of lymphatic invasion of tumor cells is an important prognostic factor related to survival and recurrence of this disease in the upper and lower urinary tract (37-39), it is suggested that the type and histological grade may also have a prognostic predictive value, and should always be carefully evaluated and considered in establishing treatment for canine bladder UC cases. More studies should be carried out in order to confirm and investigate the predictive potential of this histological variables and the biomarkers Gata-3 and Caveolin-1 for canine bladder UC, preferably also correlating them with overall survival.

5. Conclusions

GATA-3 and Caveolin-1 were demonstrated as potential biomarkers of the biological behavior of canine bladder carcinoma, correlating with mitotic count. The histopathological type and grade were also considered important factors to be evaluated carefully at the time of diagnosis as potential predictors of this tumor behavior, correlating with the lymphatic invasion. More studies are necessary to continue to investigate the

meaning of the Ki67, GATA-3 and Caveolin-1 expression for de canine bladder UC, preferentially associating those also with overall survival.

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