UNIVERSIDADE FEDERAL DE MINAS GERAIS
Faculdade de Medicina
Programa de Pós-Graduação em Patologia

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CARCINOMAS SÓLIDOS DA GLÂNDULA MAMÁRIA CANINA: aspectos morfológicos e imuno-histoquímicos

BELO HORIZONTE-MG

2021

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Versão final

Tese apresentada ao Programa de Pós-Graduação em Patologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Geovanni Dantas Cassali

Belo Horizonte
2021

043 Nakagaki, Karen Yumi Ribeiro.
Carcinomas sólidos da glândula mamária canina: aspectos morfológicos e imuno-histoquímicos [manuscrito] / Karen Yumi Ribeiro Nakagaki. – 2021.
96 f. : il. ; 29,5 cm.

Orientador: Prof. Dr. Geovanni Dantas Cassali.
Tese (doutorado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Programa de Pós-Graduação em Patologia.

1. Patologia. 2. Neoplasias Mamárias Animais. 3. Adenomioepitelioma. 4. Mioepitelioma. 5. Carcinoma Papilar. 6. Carcinoma Neuroendócrino. I. Cassali, Geovanni Dantas. II. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. III. Título.

CDU: 616

Ficha catalográfica elaborada pela bibliotecária Fabiane C M Reis – CRB 6 – 2680

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA DA UFMG

ATA DA DEFESA DA TESE DE DOUTORADO N° 430 DE KAREN YUMI RIBEIRO NAKAGAKI

Realizou-se, no dia 07 de maio de 2021, às 9 horas, por vídeo conferência, Plataforma online, a defesa de Tese, intitulada **"Carcinomas Sólidos Da Glândula Mamária Canina: Aspectos Morfológicos E Imunohistoquímicos"**, apresentada por Karen Yumi Ribeiro Nakagaki, número de Registro 2017668316, graduada no curso de Medicina Veterinária, como requisito parcial para a obtenção do grau de Doutor em PATOLOGIA, à seguinte Comissão Examinadora: **Prof. Dr. Fernando Carlos de Laender Schmitt,** Universidade do Porto; **Profa. Dra. Helenice Gobbi,** UFTM; **Profa. Dra. Fátima Gärtner,** Universidade do Porto; **Profa. Dra. Marina de Brot Andrade,** AC Camargo Center; **Prof. Dr. Geovanni Dantas Cassali, ICB/UFMG (ORIENTADOR)**.

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Documento assinado eletronicamente por **Helenice Gobbi**, **Usuário Externo**, em 27/07/2022, às 16:36, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

Documento assinado eletronicamente por **Marina De Brot Andrade**, **Usuária Externa**, em 13/10/2022, às 10:59, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Documento assinado eletronicamente por **Maria de Fátima Rodrigues Moutinho Gärtner**, **Usuário Externo**, em 13/03/2023, às 11:53, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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Referência: Processo nº 23072.240692/2022-01 SEI nº 1592643

Dedico este trabalho aos amores da minha vida: Meu pai Arnaldo, minha mãe Vera (in memoriam), minha irmã Keiko e ao meu companheiro Silas.

AGRADECIMENTOS

A Deus, por ter abençoado todos os dias da minha vida, por iluminar meu caminho e me dar forças para seguir sempre em frente.

A minha mãe Vera (*in memoriam*) minha grande fonte de inspiração. Obrigada por ter lutado tanto pela minha formação pessoal e profissional. Tudo que faço é para que eu possa ser um pouquinho dessa grande mulher que você foi! Já sinto seu sorriso orgulhoso por mais essa etapa concluída!

A minha irmã e melhor amiga Keiko e ao meu pai Arnaldo, minhas fortalezas! Obrigada por sempre apoiarem minhas escolhas, por muitas vezes abdicarem de suas vidas em favor da minha e por entenderem minhas ausências nesses últimos anos de trabalho e estudo. Eu não seria nada sem vocês!!!

Ao amor da minha vida Silas, meu companheiro, amigo e grande incentivador. Teria sido impossível sem sua presença diária, seu abraço acolhedor ao final de cada dia. Sou muito grata por ter você na minha vida e por tudo que construímos juntos! Te amo!

Ao professor Geovanni Cassali, grande amigo e orientador. Tenho muito orgulho de citá-lo como um dos responsáveis pela minha formação profissional. Agradeço pela confiança, pela amizade, risadas, desabafos, conselhos, paciência e oportunidades. Você é um exemplo de generosidade e competência. Seus ensinamentos vão além da patologia, levo comigo para vida. Além de tudo é um grande chefe! Obrigada pela bons vinhos e excelentes pratos que compartilhamos juntos.

Aos meus alunos de iniciação científica, Iara, Pedro e principalmente a Maíra, por ter sido meu braço direito nesse projeto! Tenho muito orgulho da profissional que se tornou e serei eternamente grata por esses anos de convivência e dedicação.

A todos os colegas do LPC, pelo carinho, convivência harmoniosa e por estarem sempre dispostos a ajudar.

A minha equipe do Celulavet, Marcella, Patrícia, Érica, Igor, Maíra, Úrsula, Rafa e Gabriel. Aprendo todos os dias com vocês o quanto é importante trabalhar em equipe, ser tolerante e respeitar as particularidades de cada um. Agradeço por tornarem meu dia a dia mais leve e prazeroso.

A minhas amigas “VIDA LOKA” Lívia, Vanessa e keiko, pelas trocas e desabafos diários! Por toda energia, incentivo e alegria que trazem pra minha vida. Vocês foram “meus momentos de descanso” nessa grande etapa da minha vida.

A minha amiga e exemplo de patologista Camila. Agradeço sua parceria e disponibilidade diária nessa nossa missão de diagnosticar, por estar sempre presente na minha rotina e na minha vida. Obrigada por ser minha “dupla patológica”!

A minha afilhada Bibi, aos compadres Ki e DJ pela amizade, carinho e momentos de diversão!

A minha família e amigos que sempre torceram pelo meu sucesso!

Aos pacientes, tutores e veterinários, que tornaram esse trabalho possível.

A banca pela disponibilidade e contribuições.

À CAPES, CNPq e UFMG, por ter permitido todo o trabalho possível.

Gratidão a todos vocês!!!

Resumo

Os tumores de mama são as neoplasias mais frequentes na cadela e representam um grupo heterogêneo em termos de morfologia e comportamento biológico. Cerca 50% são considerados malignos e, diante de tal fato, muitas tentativas têm sido realizadas para melhorar a classificação histopatológica, a fim de avaliar com mais precisão o seu comportamento biológico. O objetivo desse trabalho foi relatar o primeiro caso de carcinoma basaloide na glândula mamária canina, descrever as características morfológicas e imuno-histoquímicas do carcinoma neuroendócrino na cadela e propor uma subclassificação dos carcinomas sólidos e correlacioná-las com fatores prognósticos, características morfológicas e imuno-histoquímicas. Foram selecionados 135 casos de carcinoma mamário sólido, de 1800 neoplasias mamárias caninas encaminhadas ao Laboratório de Patologia Comparada-ICB/UFMG entre os anos de 2011 a 2018. Foi realizada revisão histopatológica dos casos, obtidos dados de sobrevida e realizada técnica de imuno-histoquímica de casos selecionados para os marcadores: cromogranina A, sinaptofisina, NSE, PGP 9.5, CD56, pancitoqueratina, citoqueratina 14, Ki67 e p63. Os carcinomas sólidos foram classificados em seis subgrupos determinados pelas características morfológicas e imuno-histoquimicas, sendo eles o adenomioepitelioma maligno (68/135), carcinoma padrão sólido (22/135), mioepitelioma maligno (16/135), carcinoma basaloide (14/135), carcinoma neuroendócrino (10/135) e carcinoma papilar sólido (05/135). Tempo de sobrevida mais curtos foram associados a presença de invasão linfática ($p=0,009$) nos estadiamentos clínicos iniciais (I-III). Quando considerados todos estádios clínicos (I-V), a invasão vascular ($p<0,001$) e presença de metástase regional ($p=0,004$) foram importantes fatores prognósticos. O mioepitelioma maligno apresentou a maior mediana de sobrevida em relação aos demais tipos em estádio inicial e avançado. O carcinoma invasor foi relacionado ao maior número de metástases regionais. Apesar dos carcinomas sólidos da mama canina apresentarem um mesmo padrão de arranjo celular, demonstram características morfológicas e imunofenotípicas distintas. Dessa forma, se torna imprescindível a distinção desses novos tipos histológicos, com melhor entendimento do comportamento biológico e determinação do prognóstico, para escolhas de terapias específicas e adequadas.

Palavras-chave: adenomioepitelioma maligno, carcinoma basaloide, mioepitelioma maligno, carcinoma papilar sólido, carcinoma invasor, carcinoma neuroendócrino.

Abstract

Mammary neoplasms are the most common neoplasms in the dog and represent a heterogeneous group in terms of morphology and biological behavior. Approximately 50% are considered malignant and, given this fact, many attempts have been made to improve the histopathological classification, in order to more accurately assess their biological behavior. The objective of this study was to report the first case of basaloid carcinoma in the canine mammary gland, as morphological and immunohistochemical characteristics of neuroendocrine carcinoma in bitch and propose a subclassification of solid carcinomas and to correlate them with prognostic factors, morphological and immunohistochemical characteristics. 135 cases of solid mammary carcinoma were selected, among 1800 canine mammary neoplasms referred to the Laboratory of Comparative Pathology-ICB / UFMG between the years 2011 to 2018. Histopathological review of the cases was carried out, survival data were obtained and immunohistochemistry technique was performed of cases selected for the markers: chromogranin A, synaptophysin, NSE, PGP 9.5, CD56, pancitokeratin, cytokeratin 14, Ki67 and p63. Solid carcinomas were classified into six subgroups determined by morphological and immunohistochemical characteristics, being those malignant adenomyopithelioma (68/135), carcinoma with solid pattern (22/135), malignant myoepithelioma (16/135), basaloid carcinoma (14/135), neuroendocrine carcinoma (10/135) and solid papillary carcinoma (05/135). Shorter survival time was associated with presence of lymphatic invasion ($p = 0.009$) in the initial clinical staging (I-III). When considering all clinical stages (IV), vascular invasion ($p < 0.001$) and presence of regional metastasis ($p = 0.004$) were important prognostic factors. Malignant myoepithelioma had the highest median survival in early and advanced stages. Invasive carcinoma was associated with a greater number of regional metastases. Despite the solid carcinomas of the mammary gland showing the same pattern of cellular arrangement, they demonstrate distinct morphological and immunophenotypic characteristics. Thus, it is essential to distinguish these new histological types, with a better understanding of biological behavior and determination of the prognosis, for the choice of specific and appropriate therapies.

Keywords: malignant adenomyopithelioma, basaloid carcinoma, malignant myoepithelioma, solid papillary carcinoma, invasive carcinoma, neuroendocrine carcinoma.

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LISTA DE ABREVIATURAS

AME	Adenomioepiteliomas
CA	Califórnia
CEUA	Comissão de Ética no Uso de Animais
CI	Confidence interval
CK	Citoqueratina
CK 14	Citoqueratina 14
CK 17	Citoqueratina 17
CK 5	Citoqueratina 5
CK 8	Citoqueratina 8
CK18	Citoqueratina 18
CK19	Citoqueratina 19
CK7	Citoqueratina 7
CNE	Carcinoma neuroendócrino
CPS	Carcinoma papilar sólido
DAB	Diaminobenzidina
ER	Estrogen receptor
HR	Hazard ratios
IBC NST	Invasive breast carcinomas of no special type
ICB	Instituto de Ciências Biológicas
IHC	Immunohistochemistry
NEC	Neuroendocrine carcinoma
NET	Neuroendocrine tumor
NSE	Neuron specific enolase
OMS	Organização Mundial da Saúde
PGP 9.5	Protein gene product 9.5
PR	Progesterone receptor
SMA	Smooth Muscle Actin
SS	Specific survival
TNE	Tumor neuroendócrino

TNM	Tumor-Node-Metastasis
TX	Texas
UFMG	Universidade Federal de Minas Gerais
USA	United States of America
WHO	World Health Organization

SUMÁRIO

1. INTRODUÇÃO

1.1 Neoplasias mamárias caninas

Neoplasias mamárias são os tumores mais frequentes em cadelas não castradas, representando de 50-70% de todas as neoplasias que ocorrem nesta espécie (MOULTON et al., 1970; MOE 2001; FERREIRA et al., 2003; MERLO et al., 2008).

Os tumores mamários se manifestam como nódulos únicos ou múltiplos dentro da glândula mamária e podem ser detectados durante a palpação de todos os 5 pares de glândulas (FOWLER et al., 1974; BENJAMIN et al., 1999). Vários tumores são frequentemente observados numa única glândula mamária ou pode envolver múltiplas glândulas simultaneamente e podem ser de diferentes tipos histológicos. No entanto, o tumor com o pior prognóstico sempre determina a evolução clínica do paciente. As glândulas mamárias abdominais caudais e inguinais são acometidas com mais frequência do que as glândulas torácicas (CASSALI et al., 2014).

No Brasil, a ocorrência de neoplasias mamárias malignas em cadelas pode variar de 60-90% de totas neoplasias mamárias na espécie (OLIVEIRA et al., 2003; TORÍBIO et al., 2012, NUNES et al., 2018). O comportamento biológico dessas neoplasias é variável, sendo o prognóstico influenciado por diversos fatores como: idade, tipo histológico, estadio clínico, tamanho tumoral, comportamento biológico do tumor, índice mitótico, grau histológico, metástase regional ou à distância e marcadores moleculares (SORENMO, 2003; CASSALI et al., 2014; CASSALI et al., 2020).

Os tumores mamários caninos são morfológicamente e biologicamente heterogêneos, o que tem levado a várias tentativas de classificá-los com base em suas características histopatológicas, visto sua importância em predizer o comportamento biológico do tumor (BOSTOCK, 1986; SORENMO et al., 2013; CASSALI et al., 2014; IM et al., 2014). Essa classificação é realizada a partir da avaliação de várias características tumorais como diferenciação celular, componente celular envolvido, arranjo celular e invasão da membrana basal. (CASSALI et al., 2014).

1.2 Carcinomas sólidos da glândula mamária canina

Dentre os tipos histológicos descritos, o carcinoma sólido é um padrão comum de tumor de mama em cães. Em um levantamento epiteliológico recente foi encontrada uma prevalência de 8,06% de casos de carcinoma sólido, dentre 1310 neoplasias malignas da mama da cadela. Neste estudo, somente o carcinoma em tumor misto apresentou maior número de casos, representando 44,18% da amostragem total (NUNES et al., 2018).

O que caracteriza morfologicamente esse tipo histológico é o arranjo denso das células epiteliais, sustentadas por um estroma escasso ou inaparente, com ninhos de células invasoras formando massas sólidas, com raras formações tubulares. As células são poligonais a ovais e frequentemente têm limites celulares mal demarcados e citoplasma escasso, que pode ser ligeiramente eosinofílico a basofílico. Os núcleos são redondos a ovais e frequentemente hipercromáticos com cromatina grosseiramente pontilhada e nucléolo evidente e por vezes nucléolos múltiplos. A anisocariose e a anisocitose são moderadas a acentuadas e o número de mitoses é variável. A infiltração de células neoplásicas nos vasos linfáticos pode ser encontrada, assim como metástases para linfonodos regionais. (GOLDSCHMIDT et al., 2011; CASSALI et al., 2014).

Ele apresenta prognóstico ruim de acordo com estudos de sobrevida em cadelas. Rasotto et al. (2017) demonstraram em seu trabalho que cadelas apresentando carcinoma sólido tiveram tempo médio de sobrevida de 8 meses. Apenas os carcinomas anaplásicos e carcinossarcomas tiverem tempo de sobrevida menor, de 3 meses. Resultado semelhante foi observado por Nunes et al. (2018), que demonstrou mediana de sobrevida de 268 dias para o carcinoma sólido, sendo essa sobrevida maior apenas que o carcinoma micropapilar (120 dias) e o carcinossarcoma (113 dias).

1.3 As células mioepiteliais

Os carcinomas mamários caninos e felinos têm várias características em comum com o carcinoma da mama humano, desde a epidemiologia até o comportamento clínico e

características prognósticas (DE LAS MULAS; REYMUNDO, 2000; DE LAS MULAS et al., 2004). No entanto, existem algumas diferenças entre as três espécies, principalmente no que diz respeito à morfologia. Na espécie felina e humana, os carcinomas mamários são geralmente simples, carcinomas do tipo célula epitelial luminal (MISDORP et al.,1999; SORENMO et al., 2011) enquanto na espécie canina, os carcinomas podem também ser compostos por componentes epiteliais luminais, mioepiteliais e mesenquimais (MISDORP et al., 1999; DE LAS MULAS et al., 2004).

Nas glândulas mamárias caninas e humanas normais, ductos e lóbulos são revestidos por 2 camadas de células, uma população interna luminal de células secretoras e uma camada celular externa distinta, justaposta à membrana basal, denominada camada basal / mioepitelial. Em ambas as espécies, as células epiteliais luminais são caracterizadas pela expressão de citoqueratinas (CKs) luminais de baixo peso molecular, incluindo CK8, CK18, CK19 e CK7. A camada celular externa é formada por células que expressam variavelmente CKs basais de alto peso molecular, como CK5, CK14 e CK17, além de outros marcadores, como actina de músculo liso (SMA), calponina e p63 (DEUGNIER et al., 2002; GUDJONSSON et al., 2005; SORENMO et al., 2011).

A função mais óbvia e importante da célula mioepitelial na mama é a contração do ducto da glândula mamária. As células mioepiteliais estão ligadas às células luminais e controlam muitos aspectos das funções luminais. Eles regulam o fluxo de fluido e controlam a entrada e saída de nutrientes, eletrólitos e outros fatores de crescimento. As células mioepiteliais da mama humana têm a propriedade de autorrenovação e consistentemente sofrem proliferação e diferenciação para substituir células mioepiteliais danificadas, envelhecidas ou mortas (PANDEY et al., 2010).

Nas últimas décadas, a investigação de marcadores de diferenciação celular tem sido utilizada na medicina veterinária principalmente para aprimorar nosso conhecimento da histogênese dos tumores mamários caninos. Existem hipóteses conflitantes sobre o papel do mioepitélio na gênese dos tumores complexos e mistos, as neoplasias mamárias mais comuns em cães. (SORENMO et al., 2011).

A expressão de calponina, SMA e p63 foi avaliada por diversos estudos e o p63 foi considerado mais específico para células mioepiteliais do que as proteínas contráteis calponina e SMA, devido à ausência de qualquer reação cruzada com miofibroblastos estromais (PANDEY et al., 2010; SORENMO et al., 2011). As citoqueratinas basais (CK5 e CK14) marcam as células mioepiteliais e não marcam os fibroblastos do estroma, mas têm uma especificidade baixa porque algumas células epiteliais (normais e neoplásicas) expressam citoqueratinas do tipo basal (PENÃ et al., 2014).

Muitas linhas de evidência sugerem que as células mioepiteliais diferenciadas são "supressoras naturais de tumor" porque inibem a proliferação em células de carcinoma mamário, induzindo parada de crescimento e apoptose, além de inibir a angiogênese. (STERNLICHT et al., 1997; DEUGNIER et al., 2002). Esse efeito supressor das células mioepiteliais, provavelmente depende de sua diferenciação completa e mudanças no seu padrão de expressão gênics pode levar a uma reversão de sua função, ou seja, células mioepiteliais indiferenciadas podem promover progressão tumoral em vez de suprimi-la. (CASSALI et al., 2017). No entanto, os mecanismos moleculares subjacentes às funções das células mioepiteliais durante a progressão do tumor ainda não são claros (PENÃ et al., 2014).

A identificação de células mioepiteliais em cortes de tecido corados rotineiramente é geralmente fácil em tumores complexos e mistos do cão devido à sua aparência morfológica. Elas têm formato fusiforme ou, menos frequentemente, poligonal, formam ninhos ou fascículos e estão entremeadas em uma matriz intercelular de coloração clara. (DE LAS MULAS et al., 2004). No entanto, essas células podem apresentar diferenças marcantes em sua morfologia, variando de células fusiformes a células redondas e poligonais (PENÃ et al., 2014).

Em contraste com os cães, a proliferação mioepitelial é incomum no câncer de mama humano. Os tumores da mama canina que mostram diferenciação para células mioepiteliais, como adenomioepitelioma ou mioepitelioma maligno puro são raros e geralmente têm um padrão de crescimento de células predominantemente fusiformes (PENÃ et al., 2014).

1.4 Adenomioepiteliomas malignos e mioepiteliomas malignos

Os adenomioepiteliomas (AMEs) da mama humana são tumores bifásicos que podem afetar a pele, as glândulas salivares e a mama. Na histologia, os AME são encontrados com arranjo predominantemente sólido, mas eles podem exibir áreas tubulares ou papilares. Independentemente do padrão de crescimento, dois tipos de células são observados. (FOSCHINI; EUSEBI,1998; LEE et al., 2019; GINTER et al., 2020).

Com base na classificação de tumores da mama da Organização Mundial da Saúde (OMS) humana, as lesões mioepiteliais são compostas por uma população pura ou dominante de células mioepiteliais, enquanto as lesões epiteliais-mioepiteliais são derivadas de uma população dupla de células epiteliais e mioepiteliais. As lesões epiteliais mioepiteliais incluem adenomas pleomórficos, adenomioepiteliomas (AMEs), AME com carcinoma e carcinomas adenoides císticos (ITO et al., 2019).

De acordo com a quinta edição da classificação da OMS humana, os adenomioepiteliomas malignos são os adenomioepiteliomas com carcinoma, no qual o componente maligno pode ser qualquer um dos componentes epiteliais luminas, mioepiteliais, ou ambos. Quando os dois componentes são considerados malignos, se denomina “carcinoma epitelial-mioepitelial” (FOSCHINI et al. 2019).

O mioepitelioma maligno puro é um tumor composto exclusivamente por células mioepiteliais malignas. A histologia desses casos é heterogênea. Parece que os tumores com diferenciação mioepitelial podem ter características bem ou mal diferenciadas paralelas aquelas vistas em carcinomas invasivos comuns. É possível que os tumores que demonstram diferenciação mioepitelial são o resultado da transformação neoplásica de uma célula-tronco capaz de dupla diferenciação (FOSCHINI; EUSEBI,1998).
Na cadela, os mioepiteliomas malignos, são tumores originários das células mioepiteliais da mama, e são bastante raros. Em muitos casos é necessária diferenciação por imuno-histoquímica dos fibrossarcomas (SORENMO, 2003).

1.5 Carcinomas papilares sólidos

As lesões papilares da mama na mulher têm sido tradicionalmente desafiadoras para os patologistas. Elas representam um grupo complexo de lesões que variam de benignas a malignas. A terminologia e os critérios diagnósticos usados para o diagnóstico das lesões papilares têm sido amplos, aumentando a dificuldade inerente que essas lesões representam para o patologista (SAREMIAN; ROSA, 2012).

Dentro esse grupo de lesões, o carcinoma papilar sólido (CPS) constitui uma entidade distinta clínica e morfologicamente. Os CPS´s são tumores de baixo grau originados de ductos grandes ou dilatados (SAREMIAN; ROSA, 2012). Segundo a quinta edição da OMS de neoplasias mamárias em humanos, os carcinomas papilares sólidos podem ser *in situ* ou invasivos, frequentemente mostram diferenciação neuroendócrina e são biologicamente indolentes (MAC GROGAN, et al. 2019).

Na mulher, esse tipo histológico é responsável por menos de 1% dos carcinomas de mama, sendo considerado uma entidade rara que ocorre preferencialmente em mulheres idosas. Foi descrito pela primeira vez por Maluf e Koerner em 1995 e é definido como uma forma distinta do carcinoma papilífero, caracterizado por nódulos expansivos, justapostos, com núcleos fibrovasculares delicados e crescimento em padrão sólido em menor aumento, com diferenciação neuroendócrina frequente e / ou componente invasivo mucinoso (SAREMIAN; ROSA, 2012; GUO et al., 2016).

Os estudos demonstram que o carcinoma papilar sólido da mama é uma neoplasia com bom prognóstico, incluindo uma baixa porcentagem de metástase regional e a distância, um baixo nível de recorrência local e uma baixa porcentagem de morte relacionada a esse carcinoma de mamam (GUO et al., 2016; TAN et al., 2016). Em casos associados a carcinoma invasivo, o prognóstico dependerá do componente invasivo do tumor, morfologia e graduação. Nesses casos, metástases podem ocorrer (SAREMIAN; ROSA, 2012).

1.6 Neoplasias neuroendócrinas da glândula mamária

Os tumores neuroendócrinos são um grupo de neoplasias biologicamente e clinicamente heterogêneas, que se originam mais comumente nos pulmões, trato gastrointestinal e pâncreas. Embora a sua ocorrência seja rara, já foram relatados diversos casos de neoplasias neuroendócrinas na glândula mamária em humanos (NAGAHARA et al., 2016).
Este tumor foi reconhecido pela primeira vez na mulher em 1963 por Feyrter e Hartmann com base em um padrão de crescimento "carcinóide" em 2 casos de carcinoma invasivo da mama (TANG et al., 2011; OSAMURA et al., 2019). Em 2015 foi relatado o primeiro caso de carcinoma neuroendócrino originário da glândula mamária de uma cadela (NAKAHIRA et al., 2015).

Alguns carcinomas da mama humana demonstram características de diferenciação neuroendócrina. A histogênese desses tumores é debatida principalmente devido à dificuldade em localizar células neuroendócrinas em glândulas mamárias normais. A ausência de células neuroendócrinas durante desenvolvimento da glândula mamária indica que a parte neuroendócrina de um câncer mamário não se origina de um componente normal, mas que é o resultado de uma diferenciação neuroendócrina durante a progressão neoplásica (VIACAVA et al., 1995).

Na mulher, a incidência geral é de menos de 1% de todos carcinomas de mama. No entanto, a verdadeira incidência de neoplasias neuroendócrinas mamárias é difícil de avaliar, porque marcadores neuroendócrinos não são rotineiramente usados no painel imuno-histoquímico diagnóstico do câncer de mama (WACHTER et al., 2014).

A quinta edição da OMS humana categoriza cânceres de mama com diferenciação neuroendócrina em três grupos, sendo eles o carcinoma invasivo com diferenciação neuroendócrina, o tumor neuroendócrino (TNE) e o carcinoma neuroendócrino (CNE). Quando as características morfológicas neuroendócrinas e a expressão de marcadores neuroendócrinos são focais ou não são distintos o suficiente para classificar uma neoplasia como TNE ou CNE, um carcinoma invasivo com diferenciação neuroendócrina deve ser considerado (RAKHA et al., 2019).

Do ponto de vista clínico, a importância da diferenciação neuroendócrina no carcinoma invasivo de mama humana não é clara, pois alguns estudos declararam que não há valor prognóstico para sua identificação, enquanto outros mostraram que está associado a um melhor ou pior prognóstico (YUSSIF; SOLIMAN, 2018).

As características histopatológicas da diferenciação neuroendócrina incluem o arranjo de células tumorais em ninhos sólidos formados por células com núcleos redondos a ovais, com graus variáveis de atipia. Na maioria dos casos, a cromatina nuclear granular tipo “sal e pimenta” é observada, em outros, nucléolos proeminentes são evidenciados. A maioria dos tumores exibe uma quantidade moderada de citoplasma, por vezes com granulações citoplasmáticas (MOHANTY et al., 2016; YUSSIF; SOLIMAN, 2018).

Entre os marcadores celulares neuroendócrinos, a cromogranina A e a sinaptofisina são utilizadas com maior frequência e confiabilidade. Porém, marcadores neuroendócrinos geralmente não são realizados de rotina para diagnóstico e subtipo histológico do câncer de mama humano e isso pode estar correlacionado a baixa frequência de detecção desse tipo de carcinoma (NAGAHARA et al., 2016).

1.7 Carcinoma basaloide

Glândulas mamárias e glândulas salivares são glândulas tubulo-acinares exócrinas que podem se manifestar como tumores com características morfológicas semelhantes, mas que diferem em incidência e comportamento clínico dependendo se são primários nas glândulas mamárias ou salivares (PIA-FOSCHINI et al., 2003)

Adenoma e adenocarcinoma de células basais são descritos na glândula salivar humana e se caracterizam por proliferação de células formando ninhos sólidos com células centrais de citoplasma mais claro e, como característica importante, a presença de células com núcleos hipercromáticos na periferia, com arranjo em paliçada. (SEIFERT et al., 1990; WILSON et al., 2015).

As principais características histológicas para diferenciar o adenoma do carcinoma da glândula salivar são o padrão de crescimento infiltrativo, mitoses frequentes e invasão vascular nos tumores malignos. Recorrência local e metástases nos linfonodos ou pulmão também podem ocorrer (SEIFERT et al., 1990).

O adenoma basaloide da glândula mamária canina é descrito na medicina veterinária como uma neoplasia formada por células uniformes, basofílicas, dispostas em cordões e ninhos sólidos, por vezes formando trabéculas, com característica importante de células arranjadas em paliçada na periferia, ao longo da membrana basal (KWAPIEN et al., 1977; DE LAS MULAS et al., 2002). Suas características se assemelham muito ao adenoma basaloide da glândula salivar humana e o imunofenotipo deste tumor simula o desenvolvimento embrionário dos tecidos das glândulas salivares (DE LAS MULAS et al., 2002).

Sua contraparte maligna, o carcinoma basaloide da glândula mamária da cadela, foi descrito recentemente por Nakagaki et al. (2017) e apresenta características similares aos carcinomas descritos na glândula salivar humana.

2. JUSTIFICATIVA

A maioria das neoplasias mamárias caninas são malignas e podem estar relacionados a mortalidade. Diante disso, observamos uma atual preocupação dos veterinários quanto à acurácia diagnóstica dessas lesões.

A heterogeneidade morfológica intrínseca dessas neoplasias, com um envolvimento frequente de diferentes populações de células, desafia a tarefa do patologista de fornecer uma classificação precisa. Apesar dos esforços feitos nas pesquisa de tumores mamários caninos, que contribuíram para um aumento notável no conhecimento de sua biologia, muitos tipos histológicos ainda precisam ser melhor estudados e classificados.

Diante disso, vemos uma necessidade cada vez maior de melhorar a classificação dessas neoplasias, de acordo com a sua morfologia, imunofenótipo e comportamento biológico, para ter mais critérios para estabelecer o prognóstico e tratamento.

Além disso, uma terminologia como “carcinomas sólidos” para esse grupo heterogêneo de tumores, pode levar a abordagens terapêuticas equivocadas, visto que, dados importantes relativos a agressividade e comportamento biológico da neoplasia não são contemplados.

3. HIPÓTESE

Há diferenças morfológicas, imunofenotípicas e prognósticas dentro dos carcinomas sólidos da glândula mamária canina.

4. OBJETIVOS

4.1 Objetivos gerais

Avaliar os aspectos morfológicos e imunofenotípicos dos carcinomas sólidos da glândula mamária canina.

4.2 Objetivos específicos

Caracterizar os tumores sólidos através da expressão imuno-histoquímica dos marcadores moleculares e subclassificá-los de acordo com padrão de imunofenótipo. Para isso serão utilizados os seguintes marcadores: p63, pancitoqueratina AE1AE3, citoqueratina 14, marcadores neuroendócrinos (cromogranina A, sinaptofisina, NSE, PGP 9.5 e CD56) e ki67.
Correlacionar os imunofenótipos dos carcinomas sólidos com índice mitótico, invasão vascular, grau histológico, tamanho tumoral, metástase regional e tempo de sobrevida.
Relatas as características morfológicas e imuno-histoquímicas do carcinoma basaloide e carcinoma neuroendócrino da glândula mamária canina.

5. MATERIAL E MÉTODOS, RESULTADOS E DISCUSSÃO

Os tópicos material e métodos, resultados e discussão serão apresentados na forma de 3 artigos científicos, seguido de uma conclusão final.

ARTIGO 1

Não publicado, nas normas da revista *Journal of Comparative Pathology*

CANINE MAMMARY GLAND SOLID CARCINOMA: IS IT REALLY A HISTOLOGICAL TYPE OR A TUMOR CELL ARRANGEMENT?

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Abstract

Mammary neoplasms are the most frequently diagnosed tumors in the canine species and are classified into different histological types, including solid carcinomas. In this sense, the purpose of this study is to propose a subclassification of solid carcinomas and correlate these subtypes with prognostic factors, morphological and immunohistochemical characteristics. 135 cases of solid mammary carcinoma were selected among 3400 canine mammary neoplasms referred to Comparative Pathology Laboratory-ICB/UFMG. Epidemiological and survival data were obtained from the samples and immunohistochemistry labeling for chromogranin A, pan-cytokeratin, cytokeratin 14, Ki67 and p63 was performed. Solid carcinomas were classified into six subgroups determined by morphological and immunohistochemical characteristics, being those malignant adenomyopithelioma (68/135), carcinoma with solid pattern (22/135), malignant myoepithelioma (16/135), basaloid carcinoma (14/135), neuroendocrine carcinoma (10/135) and solid papillary carcinoma (05/135). Shorter survival time was associated with presence of lymphatic invasion ($p = 0.009$) in the initial clinical staging (I-III). When considering all clinical stages (IV), vascular invasion ($p < 0.001$) and presence of regional metastasis ($p = 0.004$) were important prognostic factors. Basaloid carcinoma and solid papillary carcinoma did not reach the median survival in early-stage cases and malignant myoepithelioma had the highest median survival in advanced stages. Meanwhile, carcinoma with solid pattern was associated with a greater number of regional metastases. A distinction between the different histological and immunophenotypic subtypes that present a solid arrangement through better histological and immunohistochemical criteria becomes essential for understanding the behavior of these neoplasms and the choice of more appropriate and specific therapies.

Keywords: malignant adenomyoepithelioma, basaloid carcinoma, malignant myoepithelioma, solid papillary carcinoma.

Introduction

Mammary tumors are the most frequent neoplasms in the female dog and represent a heterogeneous group in terms of morphology and biological behavior, similar to breast tumors in women (Gama *et al.*, 2003; Rasotto*et al.*, 2012). About 50% of canine mammary tumors are considered malignant, but this percentage can reach 85% in some epidemiological studies (Nunes *et al*, 2018). Considering this fact, many attempts have been made to improve the histopathological classification in order to evaluate more accurately its biological behavior (Moulton *et al.*, 1970; Misdorp *et al.*, 1972; Dutra *et al.*, 2008; Lavalle *et al.*, 2009; Cassali *et al.*, 2014).

Among the histological types described, solid carcinoma is a common pattern of mammary tumor in dogs. Some studies show that solid carcinoma represents about 8.06% of mammary lesions in dogs (Nunes *et al*, 2018). These tumors may characterize a more advanced stage of other types, since they are frequently observed when tumors develop for long periods of time without surgical intervention (Cassali *et al.*, 2014).

Solid carcinoma is morphologically characterized by dense arrangement of epithelial cells, supported by a sparse or inapparent stroma, with nests of invading cells forming solid masses with rare tubular formations. However, there are some variations in cell characteristics, raising the possibility that not all these tumors are epithelial derived (Moulton *et al.*, 1970; Goldschmidt *et al.*, 2011; Rasotto *et al.*, 2012; Cassali *et al.*, 2014). At least some solid carcinomas consist

of myoepithelial cells, which makes solid carcinoma easier to be confused with undifferentiated myoepithelioma when both tumors are highly cellular and have minimum amount of stroma (Moulton *et al.*, 1970; Misdorp *et al.*, 1972).

Several immunohistochemical markers are used to demonstrate the presence of myoepithelial cells to determine their role in the histogenesis of canine mammary tumors. However, although myoepithelial cells can be easily recognized in some types of tumors, they are difficult to be identified in other types in routine histopathological evaluation. (Gama *et al.*, 2003; Cassali *et al.*, 2012; Peña *et al.*, 2014).

Determination of cell origin is important to designate the evolution and progression of the tumor, but solid carcinomas present great immunophenotypic heterogeneity, which is difficult to be differentiated by hematoxylin and eosin. Therefore, this study aimed to evaluate the morphological and immunophenotypic aspects of solid carcinomas of the canine mammary gland and subclassify them to determine the morphological, immunohistochemical and prognostic differences of such tumor type.

Material and methods

Ethics statement

The study was approved by the Ethics Committee on Animal Use (CEUA/UFMG) under protocol number 11/2017, on June 5, 2017.

Samples

From a total of 3400 mammary neoplasms received by the Comparative Pathology Laboratory of the Federal University of Minas Gerais (UFMG), between 2011 and 2018, 125 cases of neoplasms diagnosed as solid carcinomas and 10 more cases of solid carcinoma previously confirmed by immunohistochemistry as mammary carcinomas of neuroendocrine origin were selected.

Histopathology and special staining

Representative samples of tumors were obtained, processed routinely and included in paraffin blocks. Consecutive histological sections were prepared and stained by hematoxylin and eosin routine method. Some samples were stained by Masson's special trichrome stain. Afterwards, tumor sections were evaluated and the diagnosis of solid carcinoma was defined according to the "Consensus for the diagnosis, prognosis and treatment of canine mammary tumors-2013" (Cassali *et al.*, 2014). Nottingham histological grade system was used to determine the tumor grade (Elston and Ellis, 1991).

Immunohistochemistry

Four μm thick sections of primary tumors were prepared and mounted on gelatinized slides for IHC analysis. The antigen was immunodetected by anti-mouse/anti-rabbit detection system (Novolink Polymer Detection System, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) according to manufacturer's instructions. The slides with antibodies were incubated overnight at 4° C. The antigenic recovery was in 10 mM citrate (pH 6.0) in the pressure cooker (PascalR, Dako). Endogenous peroxidase activity was blocked with 10% hydrogen peroxide solution (H2O2) in methyl alcohol. Reagents were applied manually and immunoreactivity was

visualized by incubating the slides with diaminobenzidine chromogen (DAB Substrate System, Dako, Carpinteria, CA, USA) for 3 minutes. Details of antibodies against Cromogranin A (Sorenmo *et al.*, 2019), pan-cytokeratin (Ramírez *et al.*, 2014), ki67 (Viacava *et al*., 1995), cytokeratin 14 (Sassi *et al*, 2008; Yasuno *et al*, 2013) and p63 (Fonseca-Alves *et al*, 2018; Łopuszyńskia *et al*, 2019), are shown in Table 1. Normal canine mammary gland was used as positive internal control for cytokeratin 14, pan-cytokeratin and P63. Canine adrenal gland was used as positive control for Chromogranin A. Negative controls were established using normal serum (Lab Vision Ultra V Block) instead of the primary antibody.

Table 1- Antibodies, dilutions, incubation time, temperature and methods of antigenic recovery for immunohistochemical reactions.

Antibody	Manufacturer	Clone	Dilution
Chromogranin A	Dako (California – USA)	DAK-A3	1:100
Pancitokeratin	Dako (California – USA)	AE1/AE3	1:500
ki67	Dako (California – USA)	MIB-1	1:50
CK14	Thermo Fisher (Waltham-USA)	LL002	1:800
P63	Dako (California – USA)	DAK- p63	1:100

Immunohistochemical evaluation

The cell proliferation index (ki67) was calculated by manually counting the number of positive nuclei in a total of 500 neoplastic cells using Image J software (National Institute of Health,

Bethesda, Maryland, USA). Positive or negative granular cytoplasmic expression of chromogranin was evaluated. Evaluation of cytoplasmic expression of pan-cytokeratin and nuclear expression of p63 was performed for the labeling percentage. The evaluation of cytoplasmic expression of cytokeratin 14 was qualitative and classified as positive when there was cytoplasmic labeling of neoplastic cells.

Criterion for stratification of solid carcinomas

Solid carcinomas were stratified into six subgroups, namely malignant adenomyoepithelioma, carcinoma with solid pattern, malignant myoepithelioma, basaloid carcinoma, neuroendocrine carcinoma and solid papillary carcinoma.

Cases with 10 to 90% positive nuclear labeling for p63 in immunohistochemistry, associated with the morphological pattern of cells containing cytoplasmic vacuolizations, and sometimes slightly elongated cytoplasm, were classified as malignant adenomyopitheliomas; malignant myoepitheliomas were considered when p63 labeling was greater than 90% (Yoshimura *et al*., 2014) and associated with morphological characteristics of cytoplasmic vacuolization; when there was less than 10% labeling for p63, tumors were classified as carcinoma with solid pattern. Neuroendocrine carcinomas were diagnosed when there was significant expression of chromogranin A and another neuroendocrine marker in neoplastic cells (Nakagaki *et al.*, 2021). Basaloid carcinomas were diagnosed in cases presenting morphological characteristics according to Nakagaki *et al* (2019) and positive labeling for cytokeratin 14. Solid papillary carcinomas were grouped according to their morphology: coalescent papillary projections with presence of connective axes evidenced by Masson's Trichrome stain. The summary of stratification criteria for solid canine mammary gland carcinomas are described in table 2.

Clinical follow-up

Survival data obtained from 93 patients (68.9% of the cases). The follow-up time was minimum of two years (24 months). Specific survival (SS) was estimated in days from the date of mastectomy until the date of death due to disease progression. Patients who died from a cause unrelated to mammary tumor were censored on the date of death. The start of the observation time for each individual (T_0) was defined as the date of surgery for the removal of the mammary tumor and tumor diagnosis.

Statistical Analysis

Univariate and multivariate analyzes of survival were performed to better establish the prognostic value of the variables of interest (age, tumor size, clinical stage according to the Tumor-Node-Metastasis (TNM) system established by World Health Organization (WHO) for tumors of canine mammary gland (Owen, 1980) and adapted by Sorenmo *et al.* (2013), histological grade, angiolymphatic invasion, ulceration, necrosis, mitotic index, ki67 proliferative index and immunophenotype of solid carcinomas).

Specific survival (SS) was estimated using a Kaplan-Meier analysis and the differences were compared with log-rank test. Values were considered statistically significant when $p < 0.05$. A Cox proportional hazard regression analysis was performed to identify the potential hazard ratios (HRs) associated with SS and evaluate the prognostic value of the study variables.

The multivariate analysis included only variables with P value of 0.05 or less in the univariate analysis (log-rank test). After this step, all variables of $p < 0.05$ were included in the analysis and selected through a “reverse elimination” process. The significance of the parameters of

reduced models and final model was verified through a likelihood-ratio test and the proportionality of Cox models was verified through Schoenfeld residues. The final model included all variables $p < 0.05$. Two multivariate models of Cox proportional hazards were analyzed and clinical and pathological variables were included. The first model included only cases of patients with localized disease, while the second model was performed using data from patients with local metastatic disease. All analyses were performed using Stata software, version 14.0 (Stata Corp, College Station, TX, USA).

Table 2: Criterion for stratification of solid canine mammary gland carcinomas.

Subtype	**Immunohistochemistry**	**Morphology**
Malignant adenomyopithelioma	P63: positivity between 10% to 90% of neoplastic cells	Cells with moderate-sized cytoplasm, with cytoplasmic vacuolization.
Malignant myoepithelioma	positivity in more than 90% of neoplastic cells	Cells with moderate size cytoplasm, with cytoplasmic vacuolization. In some cases, it may have slightly elongated cytoplasm.
Carcinoma with solid pattern	positivity in less than 10% of neoplastic cells	Scarce cytoplasm, high nucleus: cytoplasm ratio, round nuclei with evident nucleoli.
Neuroendocrine carcinoma	Significant expression of chromogranin A and another neuroendocrine marker in neoplastic cells	Cells with moderately sized cytoplasm, slightly eosnophilic, with varying degrees of fine granulation. Clear nucleoli and dotted chromatin, with a "salt and pepper" appearance. Some cases have cells with eosinophilic cytoplasm, without

		granulation, hyperchromatic nuclei and formation of pseudo-rosettes.
Basaloid carcinoma	Positive label for cytokeratin 14	Solid nests delimited by a layer of palisade cells on the periphery (Fig. 2A), with hyperchromatic nuclei and scarce cytoplasm. Areas of squamous metaplasia are frequently seen.
Solid papillary carcinoma	Without specific labeling	Coalescent papillary projections due to excessive growth of numerous layers of cells, supported by thin and delicate fibrovascular stroma.

Results

The 135 cases of solid carcinoma were subclassified according to table 3.

Table 3. Classification of solid carcinomas with respective number of cases and percentage.

Classification	**Case Number:**	**(%)**
Malignant adenomyoepithelioma	68	50.4
Invasive carcinoma	22	16.3
Malignant Myoepithelioma	16	11.8
Basaloid carcinoma	14	10.4
Neuroendocrine carcinoma	10	7.4
Solid papillary carcinoma	5	3.7
Total	135	100.0

Some clinical and pathological characteristics are shown in table 4. Mixed-Breed dogs (MB) represented 26% (35/135) of the sampling and Poodle was the most prevalent breed, representing 21% (28/135) of the cases. Animals over 10 years old prevailed in 61% (72/118) of the cases. The mean age of the affected animals was 11.2 years old and the average tumor size on its longest axis was 4.3 cm. Regarding histological grading, 10.3% (14/135), 46.7% (63/135) and 42.9% (58/135) were classified as grade I, II and III, respectively. Mitotic index mean, considering mitosis count in 10 High-Power Fields (40X), was 15.7 mitoses and ki67 immunolabeling mean was 40%. Of 86 cases referred with regional lymph nodes, regional metastasis was identified in 27 cases (31.4%).

Table 4. Clinical-pathological characteristics of histological subtypes of solid carcinoma.

Clinical pathological characteristics	**Histological subtypes**					
	Malignant adenomyoepithelioma	Carcinoma with solid pattern	Malignant myoepithelioma	Basaloid carcinoma	Neuroendocrine carcinoma.	Solid papillary carcinoma
Average age (years)	11.0	12.1	10.7	9.5	13.2	11.0
Average tumor size in cm (longest axis)	4.23	4.4	5.7	2.8	3.9	4.4
Presence of necrosis	87.3	100	100	81.2	100	80
Presence of vascular invasion	24.1	50	25	33.3	60	50
Regional metastasis (%)	19	62.5	20	40	25	50

Mean of mitosis figures in 10 higth-power field (40X).	16.5	12.0	13.5	12.5	27.5	14.2
Ki67 mean	36.3	39.9	34.6	30.4	67.1	62.0

Malignant adenomyoepitheliomas

Most of solid carcinomas, 50.4% (68/135) were positive for p63 in 10% to 90% of neoplastic cells, being considered malignant adenomyopitheliomas (Fig. 1A and 1B). Morphologically, the cells were organized in mantle or solid nests, presented cytoplasm that were at times scarce, at times moderate and vacuolated, with moderate pleomorphism, round nuclei and evident nucleoli (Fig. 1A). 46.6% of the cases were initially classified as grade III. The labeling pattern for p63 varied, in some cases with labeling of grouped cells and sometimes with multifocal random labeling in tumors (Fig. 1B). The mean mitotic index was 16.5 mitoses in 10 high-power fields (40X) and the mean score for ki67 was 36.3%. Of the patients in the last group, 19.0% (8/42) of the cases presented metastasis in the regional lymph node evaluated.

Carcinoma with solid pattern

Carcinoma with solid pattern (Fig. 1C and 1D) were considered when tumors were positive for cytokeratin AE1/AE3 and presented less than 10% positivity or no labeling for p63. 22 cases of carcinoma with solid pattern were obtained (16.3%) from the 135 cases studied. These tumors were characterized by cells arranged in large solid nests, with scarce fibrovascular stroma, cells with cytoplasm with indistinct borders, slightly eosinophilic round nuclei and large and evident nucleoli and moderate to severe anisocariosis (Fig. 1C). A mean of 12 mitosis figures were counted in 10 40X fields. 59% (13/22) of the carcinoma with solid pattern were graded as grade II and 41% (9/32) as grade III. In most cases, labeling for pan-cytokeratin

(AE1/AE3) was positive and diffuse (Fig. 1D) and negative for p63. 62.5% of the analyzed lymph nodes (10/16) had regional metastasis.

Malignant Myoepithelioma

Sixteen cases (12.5%) presented more than 90% labeling for p63, being thus considered malignant myoepitheliomas (Fig. 1E and 1F) using criteria of Yoshimura *et al*., 2014. In this type of tumor, cells show solid arrangement (Fig. 1E), cytoplasm with varying degrees of vacuolization and indistinct borders, sometimes slightly elongated round nuclei and evident nucleoli. A mean of 13.5 mitoses was found in 10 high-power fields (40X). Labeling for p63 was greater than 90% with diffuse distribution (Fig. 1F).

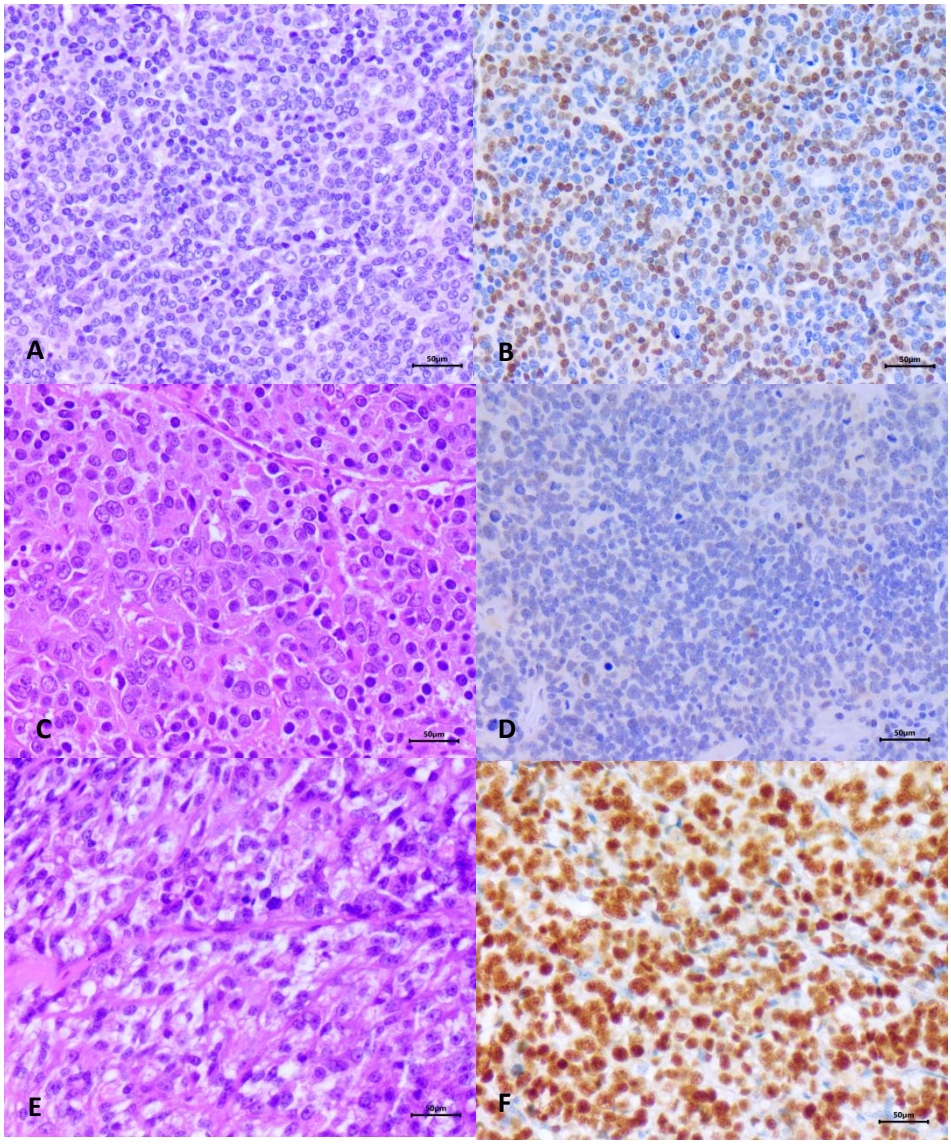

Figure 1. A) Malignant adenomyopithelioma. Cells in solid arrangement with cytoplasm of indistinct borders, round nuclei and small evident nucleoli. HE. 40X. **B) Malignant adenomyopithelioma**. Immunohistochemistry showing positive nuclear labeling for p63 in about 40% of neoplastic cells, amid cells negative for p63. 40X. C) **Carcinoma with solid pattern.** Carcinoma in a solid arrangement supported by scarce fibrous stroma. Epithelial cells showing round nuclei and moderate anisocariosis. HE. 40X. **D) Carcinoma with solid pattern.** Diffuse negative immunolabeling for p63. 40X. **E) Malignant myoepithelioma.** Carcinoma with solid arrangement forming nests delimited by delicate fibrous stroma. Cells showing cytoplasmic vacuolations in some areas. HE. 40X. **F) Malignant myoepithelioma.** Immunohistochemical staining positive for p63 in more than 90% of neoplastic cells. 40x.

Basaloid carcinoma

According to the morphological and immunohistochemical characteristics reported by Nakagaki et al. (2019), 14 (10.4%) of the 135 cases were considered basaloid carcinomas (Fig. 2A and 2B). Morphologically, cell proliferation in this histological type has a solid arrangement, sometimes multinodular, separated by moderate fibrous stroma, containing solid nests delimited by a layer of palisade cells on the periphery (Fig. 2A), with hyperchromatic nuclei and scarce cytoplasm. Anisocariosis is moderate, but it may be accentuated in some cases. A mean of 12.5 mitosis figures in ten high-power field (40X) was obtained. Areas of squamous metaplasia are frequently seen (Fig. 1A). Labeling by CK14 is observed mainly in palisade cells at the periphery of the nodules, however it may be found on much of the tumor (Fig. 1B). The immunostaining by p63 is variable, in many cases it accompanies the marking by CK14.

Neuroendocrine carcinoma

Ten cases of solid carcinomas (7.4%) were classified as neuroendocrine carcinomas based on the criteria described by Nakagaki et al. (2021). Their occurrence was low, representing 0.55% (10/1800) of all histological types in female dogs. These tumors showed an infiltrative growth pattern, with cells arranged in small solid nests delimited by delicate fibrovascular stroma. Cells presented mostly cytoplasm of moderate size, slightly eosnophilic, with varying degrees of fine granulation. The nuclei were large, round to oval, with “salt and pepper” pattern chromatin and moderate to high anisocaryosis. One of the cases showed palisade cells in some areas, often forming rosettes (Fig. 2A). Cytoplasms were scarce, slightly eosinophilic, with oval, small, hyperchromatic nuclei and single nucleoli, occasionally prominent. This morphological pattern was similar to carcinoid tumors, being classified in women as neuroendocrine tumors,

according to WHO classification of 2019 (Tan *et al*, 2020). Neoplasms in this group showed cytoplasmic labeling for chromogranin (Fig. 2B).

Solid papillary carcinoma

According to the WHO criteria for breast cancer in women, 2019 (Tan et al., 2020) and Cassali et al. (2017), five cases were classified as solid papillary carcinoma, based on morphology and Masson´s Trichrome special staining (Fig. 2E and 2F). Neoplastic proliferation in this tumor subtype is characterized by an overgrowth of papillary projections (Fig. 2E) and contains numerous layers of cells supported by thin and delicate fibrovascular stroma (Fig. 2F). Due to a large number of cell layers, the projections coalesce generating a solid aspect. Cells present moderate eosinophilic cytoplasm, with indistinct borders, large nuclei, evident nucleoli. All cases were considered invasive according to the morphology and immunohistochemical labeling for p63. Masson's special trichrome staining showed the presence of connective axes supporting epithelial cells. In this group, half of the analyzed regional lymph nodes (50%; 2/4) presented metastasis.

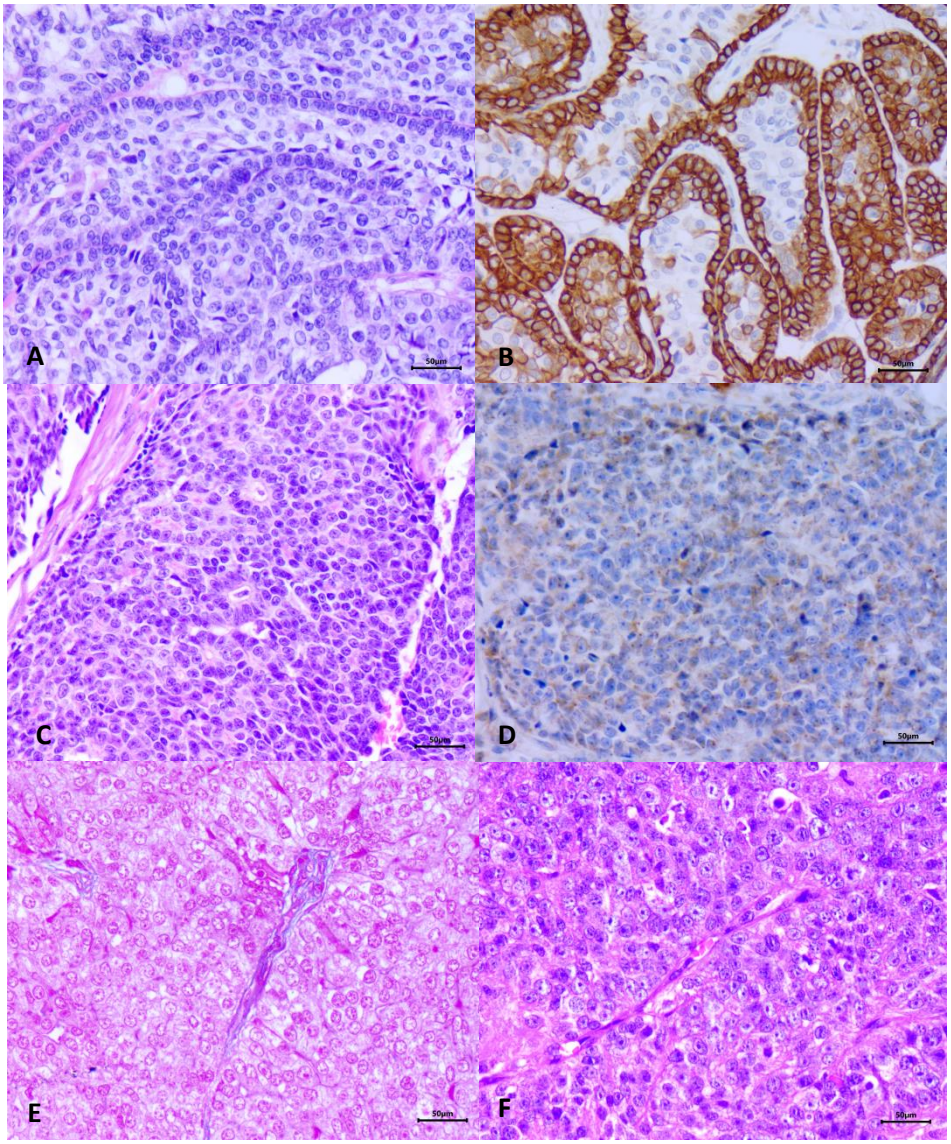

Figure 2. A) Basaloid carcinoma. Cells on the periphery of solid nests with palisade arrangement and hyperchromatic nuclei. HE. 40X. **B) Basaloid carcinoma.** Immunohistochemistry with positive cytoplasmic labeling for CK14 on palisade cells from the periphery. 40X. **C) Neuroendocrine carcinoma.** Cells with hyperchromatic nuclei with scarce cytoplasm. Areas with cells forming rosettes. 40X. HE. **D) Neuroendocrine carcinoma.** Cytoplasmic and granular immunohistochemical labeling for Chromogranin A. 40X. **E) Solid Papillary Carcinoma.** Solid nests of cells exhibiting delicate fibrovascular stromal axes, shown in blue staining. Masson's trichrome. 40X. **F) Solid papillary carcinoma**. Delicate fibrovascular axis (arrow) in the middle of epithelial cells in solid arrangement. HE. 40X.

There were no statistical differences when histological types were correlated with age, tumor size, histological grade, presence of necrosis, ulceration and vascular invasion. Correlation

between the presence of regional metastasis and histological type was statistically significant ($p < 0.05$). Metastasis was identified in 27 (31.4%) of the 86 lymph nodes analyzed. Presence of regional metastasis was greater in carcinoma with solid pattern, with 62.5% (10/16) of the cases, followed by solid papillary carcinoma with 50% (2/4), basaloid carcinoma with 40% (4/10), neuroendocrine carcinoma with 25% (1/4), malignant myoepithelioma with 20% (2/10) and malignant adenomyoepithelioma with 19% (8/42) of the cases.

In survival analysis of cases in the initial stage (I, II and III) (Figure 3), considering only the classified histological subtype, median survival of 365 days was observed for malignant adenomyopithelioma and carcinoma with solid pattern, 815 days for malignant myoepithelioma, 605 days for neuroendocrine carcinoma, while basaloid and papillary carcinomas did not reach the median ($p = 0.22$). In the univariate analysis of the variables that interfere with the survival of this early-stage model, the only factor that was statistically significant was presence of lymphatic invasion ($p = 0.009$).

In hazard ratio (HR) analysis of specific survival significant variables in model with animals in the initial stage, a higher risk of death was observed in animals with lymphatic invasion (HR = 4,09; P = 0.009, CI: 1,42-11,82).

When survival was analyzed also considering the animals in advanced stage (IV) (fig. 4), median survival of 365 days was observed for adenomyopitheliomas, 268 days for carcinoma with solid pattern, 815 days for malignant myoepithelioma, 153 days for basaloid carcinomas, 605 days for neuroendocrine carcinomas and 29 days for solid papillary carcinoma (p=0,33).

Factors interfering with the survival of this group were presence of vascular invasion (p <0.001) and regional metastasis (p = 0.004).

In hazard ratio (HR) analysis of specific survival significant variables in model with animals of all clinical stages, a higher risk of death was observed in animals with nodal metastasis (HR = 2.78; P = 0.007, CI: 1, 38-5.90).

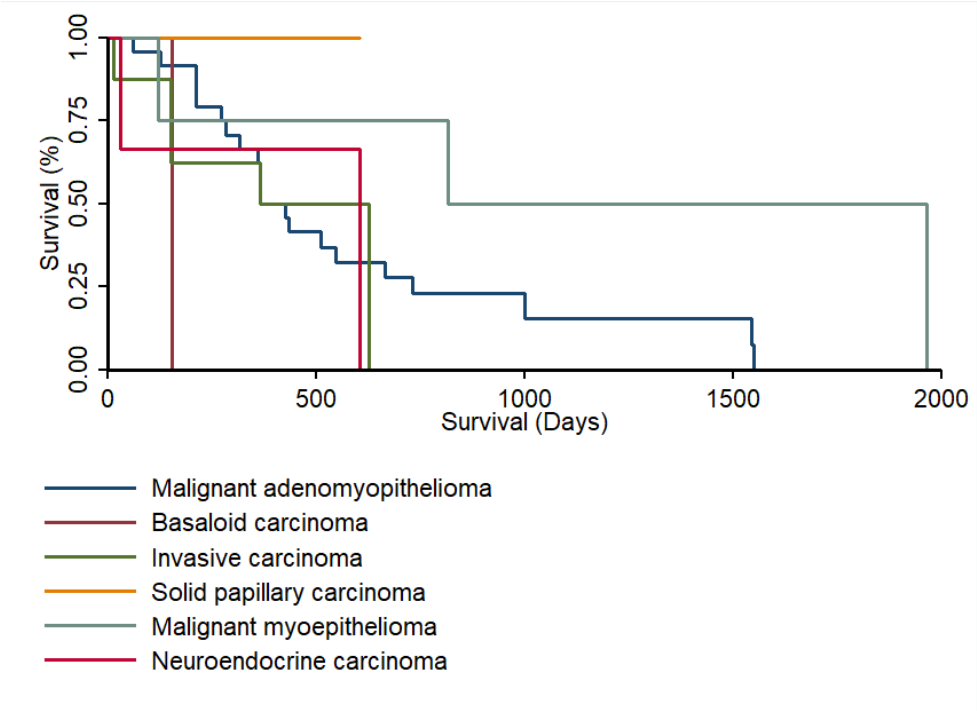

Figure 3. Kaplan-Meier curve of female dogs at early stage (I-III) of mammary tumors according to the histological subtype.

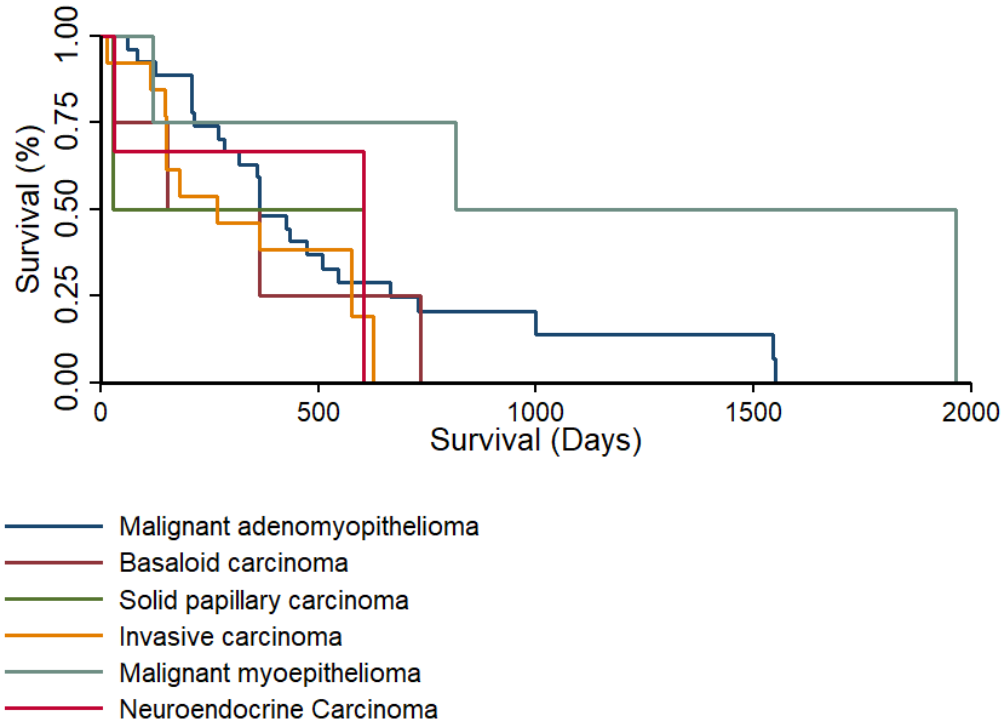

Figure 4. Kaplan-Meier survival curve of female dogs in early and advanced stages (I-V) of mammary tumors according to the histological subtype.

Discussion

Mammary tumors have been frequently diagnosed in canine species and solid carcinoma is included in the histological classification in canine mammary tumors (Misdorp *et al.*, 1972; Cassali *et al.*, 2020).

In the past, most solid carcinomas were known to comprise luminal epithelial cells, however, some studies, including the present study, have demonstrated the expression of basal/myoepithelial phenotype in this type of tumor (Yoshimura *et al*, 2014; Peña *et al*, 2014). This expression is confirmed by the marker p63, a homologue of p53, which is constantly expressed in myoepithelial cells of normal mammary glands, in hyperplasias and neoplasms. p63 is considered a sensitive and highly specific marker of myoepithelial cells, being one of its

advantages the non-expression in myofibroblasts and vascular smooth muscle (Gama *et al.*, 2003; Rosen, 2014).

Yoshimura et al. (2014) studied 72 solid carcinomas and found only 32% of the cases were composed purely by luminous epithelial cells, while 15% referred to malignant myoepitheliomas and 53% had both luminal and basal/myoepithelial epithelial components. Similar observations were described by Gama and collaborators (2003), who used p63 labeling in solid carcinomas and found that seven of the 10 analyzed cases had myoepithelial tumor cells. Our results corroborate these data, demonstrating a predominance of tumors with both epithelial and myoepithelial components.

The differentiation " basal/myoepithelial" arouses the attention of pathologists, since these tumors may exhibit more aggressive behavior, with higher rates of local recurrence and shorter survival time (Peña *et al*, 2014; Rosen , 2014). Our study demonstrated that carcinoma with solid pattern, composed predominantly of luminal epithelial cells, was a histological subtype with higher rate of regional metastasis, despite the median survival of cases in the initial stage being equal to that of malignant adenomyopithelioma. Likewise, Yoshimura et al. (2014) showed that solid canine carcinomas with myoepithelial differentiation had lower degree of malignancy than carcinomas with proliferation of luminal epithelial cells, similar to what is observed in carcinomas with basal/myoepithelial differentiation of human salivary gland.

According to WHO classification of human breast cancer in 2019, the malignant version of adenomyopithelioma is adenomyopithelioma with carcinoma, being that carcinomas may derive from luminal, myoepithelial or luminal and myoepithelial cells (Tan *et al*., 2020). In the

cases studied herein, all adenomyopitheliomas showed both luminal and myoepithelial malignant components, making it impossible to differentiate these two cell types in routine staining.

The term carcinoma with solid pattern used here is similar to invasive breast carcinomas of no special type. This group includes tumors with varied architecture, from nests, cords, trabeculae of cells and, in some cases, a solid infiltrative arrangement with scarce fibrovascular stroma. The prognosis is influenced by variables such as age, histological grade, lymphovascular invasion, as well as responses to therapeutic predictors and proliferative index by ki67 (Tan et al., 2020). In our study, this type of tumor had the highest frequency of regional metastases.

The results demonstrate that solid papillary carcinomas show an epithelial overgrowth with projections converted into solid cellular masses and are thus classified as solid tumors. Solid nests are usually arranged in a multinodular pattern amid fibrous stroma, but without fibrous capsule, with a fibro-vascular axis more delicate and difficult to be identified (Jorns, 2016). In human medicine, this histological type was first reported in 1995 by Maluf and Koerner (1995). It is a low-grade mammary tumor that originates from expanded ducts and comprises solid, well-circumscribed nodules separated by fibrovascular stroma (Lin *et al.*, 2020). As observed in the present study, the frequency of this histological type is also low in women's mammary gland, representing less than 1% of mammary carcinomas (Tan *et al*., 2020).

Regarding prognostic factors, it is important to determine whether solid papillary carcinomas may be *in situ* or invasive, but in many cases this determination is difficult, requiring identification of myoepithelial cells delimiting the nodules (Tan *et al*., 2020). Hashmi *et al*

(2020) observed in their study 60% of the cases of solid papillary carcinoma had an invasive component and only 7.5% had regional metastasis, which was considered a good prognosis by them. Other studies show metastases may occur in patients without overly invasive growth, but it is rare. The behavior when invasive carcinoma is present is consistent with its characteristics of stage and histological grade (Tan *et al*., 2020). All cases studied here showed invasive components, being that half (2/4) of the patients were already in advanced stage at the time of diagnosis, with regional metastasis and 80% (4/5) were classified as grade III. This justifies the low median survival of this histological type when there is influence of clinical stage in the analysis.

Basaloid carcinoma has recently been described in canine mammary gland and show morphological characteristics similar to those found in basaloid carcinomas of human salivary gland (Nakagaki *et al*., 2019). In this species, adenoma and basaloid adenocarcinoma represent tumors that occur mainly in the parotid gland of older individuals (Wilson and Robison, 2015). Adenomas and basaloid carcinomas have similar cytological characteristics, being composed of cells with low atypia, scarce cytoplasms and hyperchromatic nuclei, arranged in palisades on the peripheral regions of the nodules. What differs these two types and what was found in basaloid carcinomas in this study is the presence of a high proliferative index in carcinomas, in addition to frequent presence of lymphatic invasion and regional metastasis. (Atula *et al.*, 1993; Farrell and Chang, 2007; Wilson and Robinson, 2015; Nakagaki *et al.*, 2019).

Neuroendocrine carcinoma was recognized in woman's breast for the first time in 1963, by Feyrter and Hartmann (1963) and only in 2015 in the dog by Nakahira et al. (2015). This tumor occurs with low frequency in women and represents 0.5-1% of all breast cancers in this species.

As shown in the present study, the frequency in the dog is also low, representing 7.4% of solid carcinomas and 0.55% of all histological types analyzed.

As already described in the literature and observed by us, the morphological characteristics of neuroendocrine carcinoma do not define its origin in most cases, requiring specific markers, mainly chromogranin A and synaptophysin for this classification (Wachter *et al.*, 2014 ; Cloyd *et al.*, 2014; Nakagaki *et al.*, 2021). Prognosis of this histological type is not well defined in women due to its low occurrence, however, some researchers believe this may be related to the histological grade (Yussif and Soliman, 2018; Sapino *et al.*, 2021). In our study, the median survival time was 605 days, regardless of clinical staging, being the second highest median survival, only below malignant myoepitheliomas.

In our cases, a high proliferative index of tumors of all histological subtypes were found, with a mean of 15.7 mitoses in 10 high-power fields (40X) and mean of 40% for ki67. This was already expected, since a moderate to high mitotic index is observed in solid carcinomas (Misdorp *et al*, 1972; Cassali *et al*, 2014). Schmitt and Ponsa (2000) affirm that the determination of proliferation index in human breast cancer has an important prognostic impact. However, in our sample this correlation was not observed.

Morphological and immunohistochemical characteristics allowed us to classify solid carcinomas into six different subtypes. The most frequent type was malignant adenomyoepithelioma, followed by carcinoma with solid pattern, malignant myoepithelioma, basaloid carcinoma, neuroendocrine carcinoma and solid papillary carcinoma. carcinoma with solid pattern was associated with a greater number of regional metastases. Distinction between

different histological types presenting a solid arrangement through better histological and immunohistochemical criteria becomes essential to understand the behavior of these tumors and the choice of more appropriate and specific therapies.

Acknowledgments

The authors wish to thank the veterinarians and tutors who provided clinical and pathological data regarding their patients and animals. This study was financially supported by CNPQ and CAPES.

Conflicts of Interest Statement

None of the authors has any type of financial or personal conflict of interest in relation to the research, authorship or publication of this article.

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ARTIGO 2

Artigo publicado no período *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* em 14 de junho de 2019. DOI: 10.3389/fvets.2020.621714.

First description of basaloid carcinoma of the canine mammary gland: case report

[*Primeira descrição do carcinoma basaloide da glândula mamária canina: relato de caso*]

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ABSTRACT

The objective of this case report was to describe histopathological and immunohistochemical characteristics of the first reported basaloid carcinomas in the canine mammary gland. Two bitches were treated for tumors in the mammary gland and underwent mastectomy. Microscopic evaluation of these tumors revealed epithelial cells arranged in a predominantly solid pattern with hyperchromatic peripheral cells arranged in a palisade pattern. Metastases in regional lymph nodes were found in both animals, and one bitch exhibited pulmonary metastasis. Immunohistochemistry revealed positive labeling for the basal cell markers cytokeratin 14 and p63. Histopathological and immunohistochemical findings led to diagnoses of basaloid carcinoma of the canine mammary gland with regional and distant metastasis.

Keywords: mammary gland, salivary gland, basaloid carcinoma, basaloid adenoma, solid carcinoma

RESUMO

O objetivo deste relato de caso é descrever as características histopatológicas e imuno-histoquímicas do primeiro relato de carcinoma basaloide na glândula mamária canina. Duas cadelas foram atendidas com tumores na glândula mamária e foram submetidas à mastectomia. A avaliação microscópica demonstrou células epiteliais arranjadas em um padrão predominantemente sólido, com células periféricas hipercromáticas, dispostas em paliçada. As duas apresentaram metástase em linfonodos regionais e uma delas metástase pulmonar. A imuno-histoquímica revelou marcação positiva para citoqueratina 14 e p63, marcadores de células basais. Achados histopatológicos e imuno-histoquímicos levaram ao diagnóstico de carcinoma basaloide da glândula mamária canina com metástase regional e a distância.

Palavras-chave: glândula mamária, glândula salivar, carcinoma basaloide, adenoma basaloide, carcinoma sólido

Introduction

Mammary tumors are the most frequent neoplasms in bitches and represent a heterogeneous group of neoplasms with respect to morphology and biological behavior (Cassali et al., 2014). Due to the difficulty of determining the origin of a specific type of cell in certain mammary tumors, the classification of canine mammary neoplasms is based mainly on histopathological patterns (descriptive morphology) and, to a lesser extent, on histogenetic classification (Gama et al., 2003).

In human medicine, basaloid carcinoma has been described in the salivary gland and is morphologically similar to its benign variant, basaloid adenoma; however, basaloid carcinoma exhibits an invasive growth pattern (Atula et al., 1993). In veterinary medicine, basaloid adenoma has been described in the mammary glands of dogs, with histological characteristics similar to those of basaloid adenoma in the human salivary gland (Kwapien et al., 1977; De Las Mulas et al., 2002).

Although basaloid adenoma in the canine mammary gland has been well characterized, its malignant variant has not previously been reported. Therefore, the purpose of this study was to describe the morphological and immunophenotypic characteristics of two cases of canine mammary basaloid carcinoma.

Case report

Tissue specimens from two bitches were collected in the laboratory of comparative pathology of Universidade Federal de Minas Gerais to perform histopathological examinations. The first animal (case 1) was a bitch of no definite breed and exhibited a mammary tumor with the clinical attributes of inflammatory carcinoma and metastasis in an inguinal lymph node and a lung. The second animal (case 2) was a Dachshund bitch with neoplasms in the right cranial abdominal mammary gland and right axillary lymph node.

Histopathological evaluation of the primary tumors revealed neoplastic masses composed of epithelial cells arranged in solid nests that varied in size and shape; these masses were delimited by thin connective stroma. The tumor cells had scant cytoplasm; the nuclei of the centrally

arranged cells were slightly pale, and those of the hyperchromatic peripheral cells were arranged in a palisade pattern (Figure 1). Approximately three mitoses were observed in 10 fields at high magnification (40×) in both cases. In case 1, there were extensive areas of necrosis and fibrosis in addition to multifocal lymphatic invasion. Moderate fibrosis and squamous metaplasia were observed in case 2. In accordance with the classification proposed by Elston and Ellis (1998), the histological grade of the primary tumors was categorized as grade II.

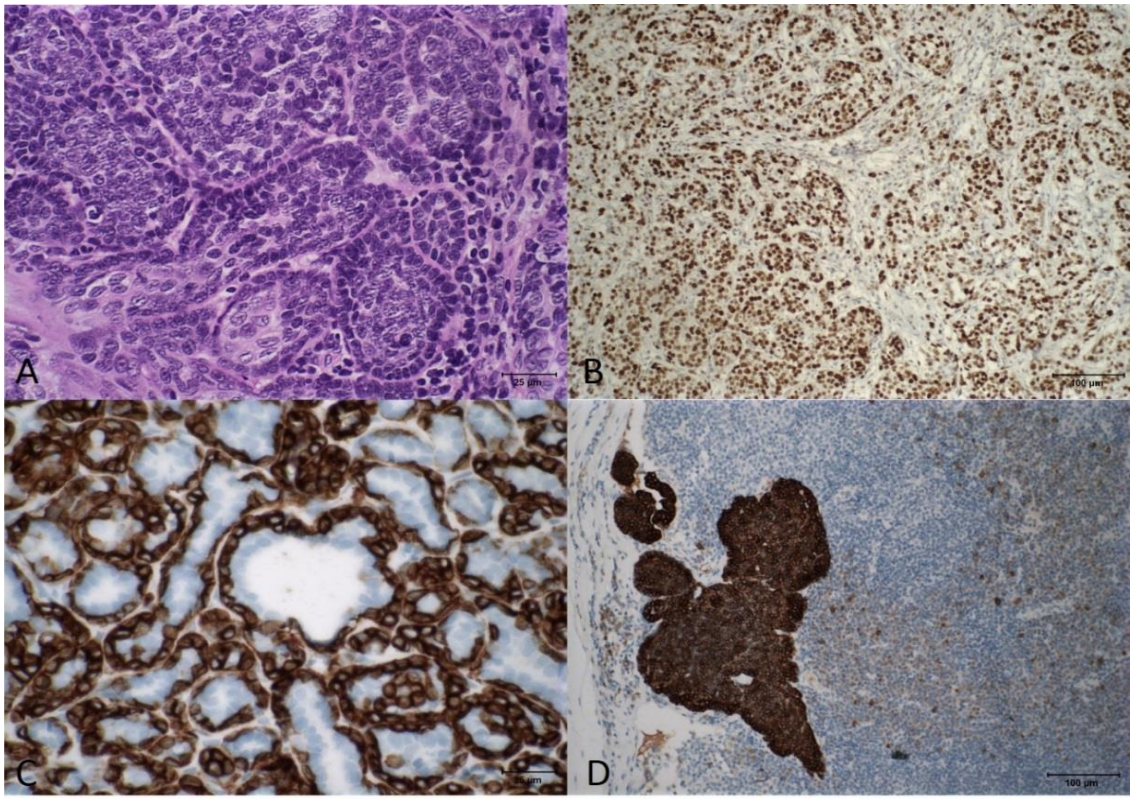

Figure 1. Bitch. Basaloid carcinoma. A. Mammary gland. Neoplastic epithelial cells arranged in solid nests with peripheral cells with hyperchromatic nuclei arranged in a palisade pattern. HE. B. Mammary gland. Immunohistochemical staining showing strong labeling for the cell proliferation antigen Ki67. Immunohistochemistry. C. Mammary gland. Primary tumor peripheral cells with intense positivity for cytokeratin 14. Immunohistochemistry. D. Lymph node showing epithelial proliferation, as evidenced by positive immunostaining for cytokeratin 14. Immunohistochemistry.

Histopathological evaluation of regional lymph nodes demonstrated macrometastasis in case 1 and micrometastasis in case 2; these metastases were characterized by epithelial cells forming solid nests, with palisade cells in the periphery. Pulmonary metastasis was observed in case 1 and was characterized by multifocal areas with coalescent malignant epithelial proliferation associated with necrotic foci, vascular invasion, and marked diffuse edema.

Histological sections of the primary tumors and the affected lymph nodes and lung were prepared for immunohistochemical analysis. A case of canine mammary basaloid adenoma was used for comparative purposes. Details regarding this analysis (Table 1) and its results (Table 2) are presented below.

Histopathological and immunohistochemical findings led to diagnoses of basaloid carcinoma with regional lymph node metastasis in both cases and pulmonary metastasis in case 1.

Table 1. Target antigens and clones, dilutions, antigen retrieval methods, and incubation times and temperatures for immunohistochemical staining for Ki-67, transformation-related protein 63 (p63), cytokeratin 7, cytokeratin 8, and cytokeratin 14

Target Antigen (Clone)	Dilution	Antigen Retrieval Method	Incubation Time (h)/Temperature
KI-67 (MIB-1)	1:50	Pressurized heat (125°C/2 min) with citrate buffer, pH 6.0	Overnight (18h)/4°C
p63 (4A4)	1:100	Water bath (98°C/20 min) with citrate buffer, pH 6.0	Overnight (18h)/4°C
CK7 (OV-TL 12/30)	1:100	Automated mode on the Benchmark® platform	30min/25°C
CK8 (TS1)	1:100	Automated mode on the Benchmark® platform	30min/25°C
CK14 (LL002)	Ready to use	Automated mode on the Benchmark® platform	30min/25°C

Table 2. Immunohistochemical results for Ki-67, p63, cytokeratin 7, cytokeratin 8, and cytokeratin 14 for basaloid tumors.

Neoplasm	KI-67 (%)	p63 (+/-)	CK7 (+/-)	CK8 (+/-)	CK14 (+/-)
Basaloid adenoma	5%	+	-	-	+
Case 1. Basaloid carcinoma	45%	+	-	-	+
Case 1. Basaloid carcinoma – macrometastases in lymph nodes	75%	+	-	-	+
Case 1. Basaloid carcinoma - lung metastases	50%	+	-	-	+
Case 2. Basaloid carcinoma	10%	+	-	-	+
Case 2. Basaloid carcinoma – micrometastases in lymph nodes	10%	+	-	-	+

Discussion

Mammary salivary glands are exocrine tubular acinar glands that can develop tumors that have similar morphological characteristics but differ in incidence and clinical behavior (Pia-Foschini et al., 2003).

Basaloid adenoma of the canine mammary gland closely resembles basaloid adenoma of the human salivary gland, and the immunophenotype of this tumor mimics that observed during the embryonic development of salivary gland tissues. Comparisons appear to indicate that basaloid proliferations of the mammary gland are relatively uncommon in humans and animals (Kwapien et al., 1977; De Las Mulas et al., 2002).

Basaloid carcinoma of the salivary gland is histologically similar to basaloid adenoma and is characterized by solid nests of cells and less often by trabecular or tubular structures. As observed in the two cases in this study, tumor cells have scant cytoplasm and strongly basophilic nuclei in a distinct palisade arrangement at the tumor periphery; these traits are characteristic

of this histological type of tumor (Seifert et al., 1990, Farrell and Chang, 2007, Wilson and Robinson., 2015).

The primary tumors and metastases of case 1 and case 2 exhibited a basal immunophenotype, as demonstrated by positive labeling with a cytokeratin 14 antibody; this immunophenotype has been observed for basaloid adenomas of the canine mammary gland and basaloid neoplasms of the human salivary gland (De Las Mulas et al., 2002, Pia-Foschini et al. (2003) and Nagao et al., 2012). Positive immunostaining was also observed for p63, a selective marker for basal/myoepithelial cells (Edwards et al., 2004; Nagao et al., 2012). A ductal glandular pattern was not observed given negativity for cytokeratins 7 and 8, for which positive staining is common in mammary neoplasms with this pattern (De Las Mulas et al., 2002; Pia-Foschini et al., 2003).

According to Seifert et al., (1990), the malignant potential of basaloid carcinoma is supported by local recurrence in 25% of cases and lymph node or lung metastasis in 10% of cases. Proliferative index and infiltrative growth are important features for differentiating basaloid adenoma from basaloid carcinoma. In cases 1 and 2, the proliferative indices determined using Ki-67 were 45% and 10%, respectively; the mitotic count was three mitotic figures in 10 fields at high magnification (40×) for both neoplasms.

For both analytical approaches, the proliferative indices for both neoplasms were higher than those observed for the examined basaloid adenoma, which exhibited 5% immunolabeling by Ki-67 and no mitotic figures in 10 fields at high magnification (40×). These findings were compatible with previously reported results of mean Ki-67 immunolabeling of 3.3% and 15.5% for basaloid adenomas and basaloid carcinomas of the salivary gland, respectively (Nagao et al., 2012, Wilson and Robinson, 2015). In our study, the two mammary neoplasms led to regional lymph node metastasis in both cases and pulmonary metastasis in case 1, confirming the malignant potential of these tumors.

In contrast to basaloid carcinomas of the salivary gland, tumors in the canine mammary gland of the observed histological type do not appear to exhibit a low degree of malignancy and a relatively favorable prognosis (Farrell and Chang, 2007). Further studies that include follow-

up data for patient survival are necessary to better understand the behavior of neoplasms of this new histological type.

Conclusion

Histological type is regarded as an independent prognostic factor for canine mammary neoplasms. Therefore, establishing criteria for and recognizing and reporting various histological subtypes of mammary tumors in dogs is essential for improving the appropriateness and effectiveness of treatment for such tumors.

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ARTIGO 3

Publicado na *Frontiers in Veterinary Science,* em 05 de janeiro de 2021. DOI: 10.3389/fvets.2020.621714.

Neuroendocrine Carcinomas of the Canine Mammary Gland: Histopathological and Immunohistochemical Characteristics

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Abstract

Invasive mammary carcinomas with neuroendocrine differentiation are rare in women and were reported only once in female dogs. For the present study, ten cases of solid mammary carcinoma positive for chromogramin A in immunohistochemistry were selected. Histopathological characteristics of these tumors were described and immunohistochemical evaluation was performed with chromogranin A, synaptophysin, CD56, NSE, PGP 9.5, pancitokeratin, Ki67, estrogen receptor (ER), and progesterone receptor (PR). The average animal age was 13.2 years old and the average tumor size was 4.8 cm. In total, 70% of the neoplasms were classified as grade III and 30% as grade II by the Nottingham histological grade system. High mitotic index was observed with a mean of 27.5 mitoses in 10 high magnification fields. Only one case showed typical carcinoid tumor characteristics. In addition, vascular invasion was shown in 3 tumors. All carcinomas were positive for chromogran A, while only two cases were reactive to synaptophysin. For PGP 9.2, NSE and CD56, we observed positivity of 100, 90, and 70%, respectively, in the samples, being that no tumor was positive for all the neuroendocrine markers. All neoplasms showed ER and PR in at least 10% of neoplastic cells, while Ki67 varied from 29 to 95%, with mean mitotic index of 67%. Four of the ten animals died within 1 year of the tumor diagnosis. Neuroendocrine neoplasms occur in the canine mammary gland and are propably underdiagnosed. This is due to their non-specific morphological characteristics and the low use of neuroendocrine immunohistochemistric markers the diagnostic routine. More studies are necessary to determine the prognosis of this new histological type.

Keywords: diagnosis, female dog, histological classification, solid carcinoma, neuroendocrine carcinoma, mammary cancer

Introduction

Neuroendocrine tumors are a group of biologically and clinically heterogeneous neoplasms that originate most commonly in lungs, gastrointestinal tract and pancreas (1, 2). Although their occurrence is rare, pure neuroendocrine tumors and invasive breast carcinomas with neuroendocrine features have already been reported in women (3–5), while only one case has been reported in a female dog (6).

These tumors were first recognized in women in 1963 by Feyrter and Hartmann (7) based on a "carcinoid" growth pattern seen in two cases of invasive breast carcinoma. Later, in 1977, Cubilla and Woodruff (8) described eight cases of breast cancer showing a growth pattern typical of a "carcinoid tumor," and several recent reviews have since been published (9–12).

Finally, the classification of breast tumors of the World Health Organization (WHO) in 2003 recognized neuroendocrine carcinomas of the breast as a special histological type of invasive carcinoma in which more than 50% of the neoplastic cells express at least one neuroendocrine marker (13). Next, the 2012 classification included a chapter on "Carcinomas with neuroendocrine features," in which the minimum cut-off of tumor cells with positive labeling for neuroendocrine markers was removed (9, 14).

The most recent WHO classification (2019) (15) categorizes breast cancers with neuroendocrine differentiation in three groups: (1) invasive carcinoma with neuroendocrine differentiation; (2) neuroendocrine tumor (NET); (3) neuroendocrine carcinoma (NEC). However, the neuroendocrine differentiation detected by either histochemical or immunohistochemical analysis may be seen in 10–30% of invasive breast carcinomas of no special type (IBC NST). Additionally, special types of breast cancer may also show expression of neuroendocrine markers, particularly solid papillary carcinomas and mucinous carcinomas, and should not be classified as NETs or NECs. When neuroendocrine morphologic characteristics and neuroendocrine marker expression are focal or are not distinct enough to classify a neoplasm as NET or NEC, an IBC NST with neuroendocrine differentiation must be considered. Notably, most breast cancers with neuroendocrine differentiation belong in the first group, as pure neuroendocrine tumors of the breast are exceptionally rare.

Neuroendocrine tumors of the breast correspond to an invasive neoplasm composed of densely cellular solid nests or trabeculae of cells, usually with a low to intermediate grade morphology, separated by delicate fibrovascular stroma. Papillary, insular and alveolar-like patterns may be seen. On the other hand, neuroendocrine carcinomas are invasive carcinomas characterized by the proliferation of small or large, high-grade neoplastic cells (small cell neuroendocrine carcinoma and large cell neuroendocrine carcinoma, respectively). Both subtypes present neuroendocrine morphological features, cytoplasmic neurosecretory granules and uniform immunohistochemical positivity for neuroendocrine markers (15).

Data on breast cancers with neuroendocrine differentiation are limited. Also, the true incidence of neuroendocrine neoplasms of the breast is difficult to evaluate, because many of the classic histopathological features of neuroendocrine tumors that occur in other organs are not present in their breast counterpart (13, 16). In addition, neuroendocrine markers are not routinely tested in invasive breast carcinomas, since there is no clinical relevance of neuroendocrine differentiation as an individual characteristic (12, 15).

In the female dog, the classification of mammary neoplasms is mainly based on the histopathological pattern and, to a lesser extent, on the histogenetic classification, due to the difficulty in determining the origin of a specific cell type in certain mammary tumors (17). Among the histological types described, the solid carcinoma is a common pattern of canine mammary tumor that presents some variations in cell characteristics, which makes many researchers believe that there may be several origins for these cells (18–20).

In this study, we aimed to investigate the presence of neuroendocrine differentiation in 10 cases of solid mammary carcinoma of female dogs, in order to promote a greater recognition and appropriate classification of this histological type in this species.

Materials and Methods

Ethics Statement

The study was approved by the ethics committee on animal use (CEUA/UFMG) under protocol number 11/2017, on June 5th, 2017.

Animals

Ten cases of canine mammary solid carcinoma, positive for chromogramin A in immunohistochemistry, were selected for this study. The samples were from the Laboratory of Comparative Pathology of Minas Gerais Federal University (UFMG).

Histopathology

Representative samples of tumors removed by incisional or excisional biopsy were obtained and included in paraffin blocks. Consecutive histologic sections were prepared and stained by the hematoxylin and eosin routine method. Neoplasm slides were evaluated and diagnoses were defined according to the "Consensus for the diagnosis, prognosis, and treatment of canine mammary tumors-2013" (20). The Nottingham histologic grade system was applied to determine tumor grade (21).

Immunohistochemistry

Sections of 4 μm thickness from primary tumors were prepared and mounted on common slides for IHC analysis. The antigen was immunodetected by the detection system anti-mouse/anti-rabbit (Novolink Polymer Detection System, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) according to the manufacturer's instructions. The endogenous peroxidase activity was blocked with a 10% hydrogen peroxide (H2O2) solution in methyl alcohol. Reagents were manually applied and immunoreactivity was visualized by incubating the slides with diaminobenzidine chromogen (DAB Substrate System, Dako, Carpinteria, CA, USA) for 3 min. Details of the antibodies against Synaptophysin (1, 22, 23), NSE (22, 24), CD56 (24), PGP 9.5 (25, 26), Chromogranin A (25, 27), Estrogen Receptor - RE (28), Progesterone Receptor - PR (29), Pancitokeratin (22) and Ki67 (30), dilutions, antigen retrieval procedures and incubation times used in the immunostaining process are shown in Table 1. Normal canine mammary gland was used as an internal positive control for Estrogen and Progesterone Receptors and Pancitokeratin. For Synaptophysin, NSE, CD56, PGP 9.5 and Chromogranin A, canine adrenal gland was used as positive control. Negative controls were performed using a normal serum (Lab Vision Ultra V Block) in place of the primary antibody.

Table 1: Antibodies, dilutions, incubation time and temperature and methods of antigenic recovery for the immunohistochemical reactions.

Antibody	Manufacturer	Clone	Dilution	Incubation time/ temperature	Antigenic recovery method
Synaptophysin	Monosan	SY38	1:100	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
NSE	Dako	BBS/NC/VI-H14	1:1000	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
CD56	Biocare Medical	BC56C04	1:50	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
PGP 9.5	Cell Marque	polyclonal	1:500	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
Chromogranin A	Dako	DAK-A3	1:100	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
Estrogen Receptor (RE)	Dako	1D5	1:50	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
Progesterone receptor (RP)	NeoMarkers	Ab-1 (hPRa2)	1:50	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
Pancitokeratin	Dako	AE1/AE3	1:500	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)
Ki67	Dako	MIB-1	1:50	Overnight 4°C	10mM citrate (pH 6.0) in the pressure cooker (Pascal^{R}, Dako)

Immunohistochemical Evaluation

The cell proliferation index (Ki67) was calculated by manually counting the number of positive nuclei in a total of 500 neoplastic cells in areas with the highest levels of positivity (hot spot/hot zones) through image J software (National Institute of Health, Bethesda, Maryland, USA). A 20% score was used as cutoff point to classify cases with high or low proliferation index. Positivity for estrogen receptor (ER) and progesterone receptor (PR) was defined as the

presence of nuclear expression in >10% of neoplastic cells (3). The Expression of the neuroendocrine markers chromogranin A, synaptophysin, CD56, NSE and PGP 1.9 was assessed. The evaluation pancytokeratin expression (AE1/AE3) was qualitative and classified as positive positive when there was cytoplasmic staining of neoplastic cells.

Results

The mean age of the ten studied animals was 13.2 years old, ranging from 9 to 16 years old. Mean tumor size was 4.8 cm, excluding one case of incisional biopsy for which the actual size of the neoplasm was not informed. The main pathological parameters are detaleid in Table 2.

Table 2: Macroscopic and histopathological characteristics of mammary neoplasms with neuroendocrine differentiation.

Patients	Tumor size measured to its largest extent (cm)	Mitotic figures in 10 HPFs*	Histological grade	Presence of lymphatic invasion	Regional lymph node
Patient 1	8.0	43	III	No	Not analyzed
Patient 2	1.5	46	III	Yes	Not analyzed
Patient 3	8.0	25	III	No	No metastasis
Patient 4	2.0	18	II	Yes	With metastasis
Patient 5	3.0	41	III	No	Not analyzed
Patient 6	5.0	34	III	No	Not analyzed
Patient 7	4.5	22	III	No	Not analyzed
Patient 8	7.0	22	III	No	No metastasis
Patient 9	2.0	11	II	No	No metastasis
Patient 10	4.3	13	II	Yes	Not analyzed

*HPFs, high-power fields

In general, tumors showed a high mitotic count, with a mean number of 27.5 mitoses in 10 high-power fields (40X). Seventy percent (7/10) of the neoplasms were classified as grade III and 30% (3/10) as grade II. Lymph nodes of only 4 cases were referred for analysis, of which one presented metastasis on histopathological examination.

Similar morphological patterns were observed in the histopathological analysis of all cases, with 100% demonstrating a solid cell arrangement (Figure 1A) and at least some proliferation

areas in situ. Most tumors exhibited an infiltrative growth pattern with invasion into the dermis and adjacent adipose tissue, although a circumscribed pattern was seen in some cases. Sixty percent of the cases (6/10) exhibited arrangement in small solid nests of cells delimited by delicate fibrovascular stroma (Figure 1B). Neoplastic cells presented a cytoplasm of moderate size, slightly eosnophilic, with varying degrees of fine granulation, or a clear cytoplasm, with vacuolization (Figure 1C). The nuclei were large, round to oval, with varying degrees of atypia. In addition, 60, 30, and 10% of the cases showed marked, moderate and mild anisocariosis, respectively. Nuclear chromatin was slightly dotted and dispersed (salt and pepper aspect) in most cases, and sometimes with fine condensation along the nuclear membrane. The nucleoli were either small and multiple or sometimes single and large (Figure 1D).

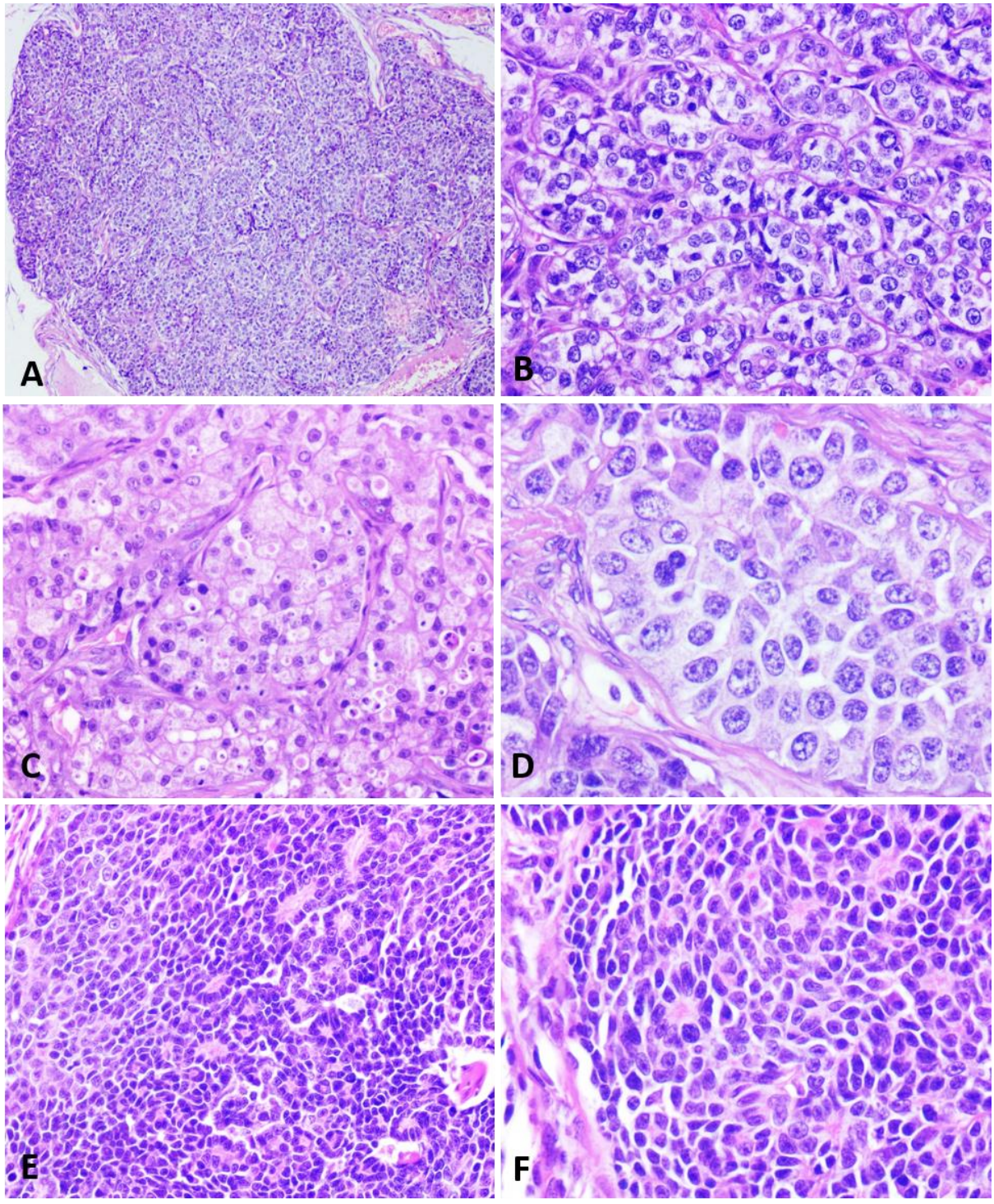

Figure 1. Histopathological characteristics of neuroendocrine carcinomas in the female dog. **(A)** A mammary lump showing a solid arrangement. Hematoxylin and eosin.10x. **(B)** Solid nests of neoplastic cells separated by a delicate fibrovascular stroma. Hematoxylin and eosin.40x. **(C)** Cells with a finely granular eosinophilic cytoplasm, sometimes displaying intracytoplasmic vacuoles. Hematoxylin and eosin.40x. **(D)** Neoplastic cells with round to oval nuclei, finely dotted chromatin, and conspicuous nucleoli. Hematoxylin and eosin.60x. **(E)** Neoplastic cells disposed in a solid arrangement, occasionally in palisades and forming rosettes. Hematoxylin and eosin.40x. **(F)** Cells exhibiting a scarce, slightly eosinophilic cytoplasm, with small and hyperchromatic nuclei. Hematoxylin and eosin.40x.

One of the neoplasms (patient 4) showed a distinct, peculiar morphology, with cells arranged in large coalescing solid nests, containing palisade cells, often forming rosettes (Figure 1E). The cytoplasm was scarce, slightly eosinophilic, with oval nuclei, small, hyperchromatic and unique nucleoli or occasionally prominent (Figure 1F). This morphological pattern was compatible with the histologic characteristics of carcinoid tumors.

Lymphovascular invasion was identified in 3 of the 10 cases (30%). Areas of necrosis, mainly at the center of solid nests, were observed in 70% of the neoplasms.

Immunohistochemistry results are shown in Table 3. All cases were positive for chromogranin A (Figure 2A) and the cytoplasmic staining was granular with variable intensity. Only two cases were positive for synaptophysin (Figure 2B). Seven tumors (7/10) were positive for CD56 (Figure 2C), while nine (9/10) were positive for NSE (Figure 2D). All cases were positive for PGP 9.5 (Figure 2E). No case was positive for all neuroendocrines.

Table 3. Expression of chromogranin A, synaptophysin, NSE, CD56, PGP 9.5, Ki67, ER, PR and pancitokeratin in solid mammary carcinomas with neuroendocrine features.

Patients	Chromogranin A	Synaptophysin	NSE	CD56	PGP 9.5	Ki67	ER	PR	CK AE1/AE3
Patient 1	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	50%	10-25%	>75%	POSITIVE
Patient 2	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	95%	51-75%	>75%	POSITIVE
Patient 3	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	75%	51-75%	>75%	POSITIVE
Patient 4	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	83%	51-75%	>75%	POSITIVE
Patient 5	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	50%	10-25%	>75%	POSITIVE
Patient 6	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	90%	25-50%	>75%	POSITIVE
Patient 7	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	89%	25-50%	>75%	POSITIVE
Patient 8	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	29%	10-25%	>75	POSITIVE
Patient 9	POSITIVE	NEGATIVE	POSITIVE	POSITIVE	POSITIVE	56%	10-25%	51-75%	POSITIVE
Patient 10	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	54%	10-25%	51-75%	POSITIVE

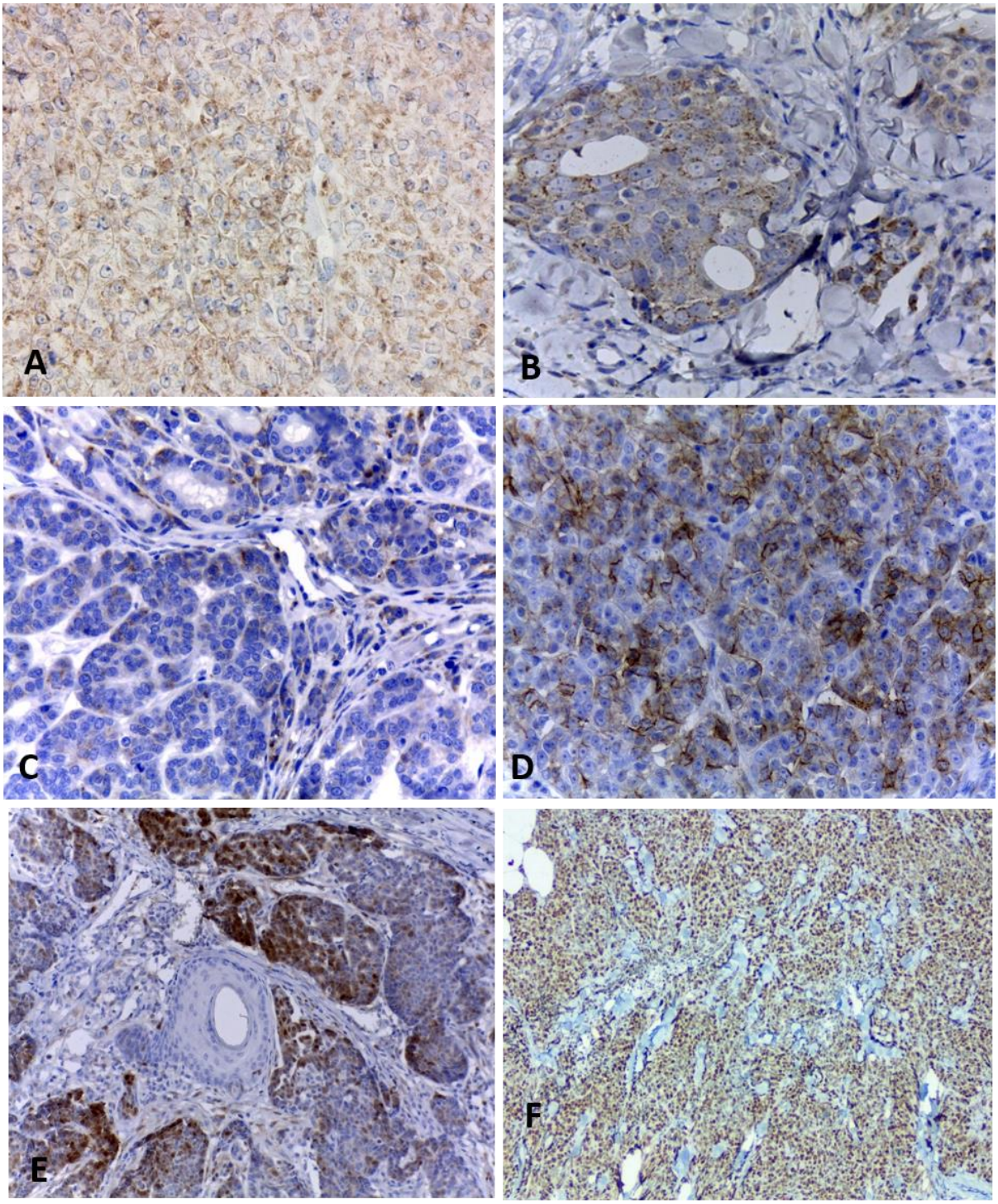

Figure 2. Immunohistochemical staining of canine mammary solid carcinomas. **(A)** Neoplastic cells showing positive cytoplasmic staining for chromogranin A, with a granular pattern. 40x. **(B)** Cytoplasmic expression of synaptophysin in more than 50% of neoplastic cells. 40x. **(C)** Less than 50% of positive neoplastic cells for CD56. 40x. **(D)** Expression of NSE in more than 50% of neoplastic cells. 40x. **(E)** Multifocal staining for PGP 9.5 in 10% of neoplastic epithelial cells. **(F)** Nuclear positivity Ki67 in 95% of neoplastic cells. 10x.

All neoplasms expressed estrogen and progesterone receptors in at least 10% of neoplastic cells and were positive for pancytokeratin (AE1/AE3). Ki67 (Figure 2F) was considered high in most carcinomas, with a mean of 67% of positive cells, ranging from 29 to 95%.

Four of the 10 studied animals (patients 1, 2, 6, and 10) died or were euthanized within 1 year of diagnosis due to the neoplasm development. Two animals (patients 3 and 4) died due to other causes unrelated to the tumor and two patients (8 and 9) are still alive with no sings of recurrence and metastasis after 1 year and 6 months and 3 years and 8 months of diagnosis, respectively. The follow-up of patients 5 and 7 after surgery was not feasible. Only one of the eight patients with follow-up (patient 10) received complementary treatment after surgery.

Discussion

Invasive carcinomas with neuroendocrine differentiation of the human breast are under-recognized in the practical routine and represent 0.5–1% of all breast cancers (11, 12, 15). In veterinary medicine, this histological type is not yet well recognized, with a single prior case report in the literature (6), making ours the first retrospective study on neuroendocrine carcinomas in the female dog.

The histogenesis of neuroendocrine tumors of the breast is debatable mainly due to the difficulty in locating neuroendocrine cells in normal mammary glands (16, 31). Viacava *et al*. (31) did not find histochemical, immunohistochemical and ultrastructural evidence of neuroendocrine differentiation in normal cells of the fetal and adult mammary glands in their study, indicating that this differentiation may happen in the process of tumor progression.

From a clinical point of view, the importance of neuroendocrine differentiation in invasive breast carcinomas is not well stablished. While some studies have stated that there is no prognostic value, others have shown that it is associated to a better or worse prognosis (14, 16, 32). Sapino *et al.* (33) concluded that the histological grade greatly influenced the clinical evolution of neuroendocrine carcinomas of the breast. Poorly differentiated, grade III neuroendocrine carcinomas with a high proliferative activity behaved aggressively. On the other hand, patients with well-differentiated, grade I tumors with a low proliferative index remained alive after more than 13 years of follow-up. Therefore, the impact of neuroendocrine

differentiation on prognosis remains unclear and may be explained by the heterogeneous nature of tumors that fall into this category, as this group includes special breast cancer types with a low-grade morphology and indolent clinical course, as well as high-grade aggressive carcinomas (32, 34). Of the eight followed-up patients, four died due to the unfavorable neoplasm clinical evolution in <1 year of diagnosis. In this sense, the fact that half of these followed-up patients survived for less than an year after diagnosis may lead to the conclusion that this type of carcinoma presents a guarded to poor prognosis compared to other carcinomas with better prognosis in female dogs, such as carcinoma in mixed tumor, which does not reach the survival median until 2 years of follow-up (35).

The diagnosis of carcinoma with neuroendocrine differentiation based solely on morphologic characterisitics is a challenge and not feasible most of the times, once many of the classic histologic features of neuroendocrine carcinomas that occur in other organs are not present in neuroendocrine carcinomas of the mammary gland (13, 16, 36). Thus, the morphologic features found in this study were similar to those described in other studies that confirmed the presence of neuroendocrine differentiation by immunohistochemistry (11, 14, 32, 37). Only one case exhibited a typical morphology of a carcinoid tumor, with smaller cells, sometimes forming rosettes, hyperchromatic nuclei and scarce cytoplasm, analogous to carcinoid tumors and well-differentiated neuroendocrine carcinomas previously reported in the human breast and other organs (38, 39). According to the WHO classification of breast tumors of 2019, this sole case should be categorized as a neuroendocrine tumor, whereas the other nine tumors should be classified as large cells neuroendocrine carcinomas according to morphologic and immunohistochemical characteristics (15).

Metastases from other neoplasms in women's breast are uncommon and metastases of neuroendocrine carcinoma are even rarer, representing 1–2% of all metastatic tumors of the breast (37). However, the possibility of metastatic neuroendocrine carcinomas should always be excluded with adequate clinical and radiological examinations as well as immunohistochemical studies to attest a breast origin in order to diagnose a primary invasive breast carcinoma with neuroendocrine features (14). The presence of an associated carcinoma in situ and positivity for hormonal receptors may also be useful for the diferential diagnosis (14, 16, 37, 40, 41). The animals studied herein had no history of neoplasms in other locations, but imaging data were not obtained. All tumors were positive for ER and PR, and had

proliferation areas in situ, corroborating the hypothesis that these were primary mammary lesions.

Among neuroendocrine differentiation markers, chromogranin A and synaptophysin are the most frequently used (1, 34). In our study, only 2 of 10 cases showed positivity for both markers and similar results have been reported by Wachter et al. (13). Such findings highlight the importance of a panel including at least two markers in cases suspected for neuroendocrine carcinoma, since these tumors will not always be positive for both antibodies.

Our findings show that neuroendocrine carcinomas occur in the canine mammary gland, as well as in the human breast and may be underdiagnosed when they are included in the group of solid carcinomas. However, a definitive diagnosis based on histopathological examination alone is challenging, stressing the need of using specific markers for neuroendocrine differentiation such as chromogranin and synaptophysin for confirmation. Thus, complementary studies with clinical and therapeutic follow-up are essential to define the prognosis of this new histological type, in addition to establishing implications in target therapy responsiveness.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics Statement

The animal study was reviewed and approved by CEUA - UFMG. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author Contributions

KN and MN collected patient data and samples from the UFMG Laboratory of Comparative Pathology file. KN, AG, and GC performed histopathological and immunohistochemical analyses. KN, GC, and MD participated in the writing of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Footnotes

Funding. CNPQ is recognized for funding the study.

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8. CONCLUSÕES FINAIS

-As neoplasias classificadas como carcinomas sólidos da glândula mamária canina apresentam diferenças morfológicas, imunofenotípicas e prognósticas e podem ser divididas em seis tipos histológicos distintos, sendo eles o adenomioepitelioma maligno, carcinoma invasor, mioepitelioma maligno, carcinoma basaloide, carcinoma neuroendócrino e carcinoma papilar sólido.

-Dentre os tipos histológicos que apresentam arranjo sólido, o que apresentou maior frequência foi o adenomioepitelioma maligno, representando 50,4% dos casos, seguido do carcinoma invasor (16,3%), mioepitelioma maligno (11,8%), carcinoma basaloide (10,4%), carcinoma neuroendócrino (7,4%) e o carcinoma papilar sólido (3,7%).

- A correlação entre o tipo histológico e as variáveis: idade, tamanho tumoral, grau histológico, presença de necrose, ulceração, invasão vascular e metástase regional só foi estatisticamente significativa para a variável metástase regional. O carcinoma invasor foi o tipo histológico com maior índice de metástase regional, com 62,5% de animais desse grupo apresentando metástase regional no momento do diagnóstico.

-Nos animais em estádio clínico inicial, a presença de invasão vascular foi a única variável que interferiu na sobrevida desses pacientes. Foi identificado risco de óbito 4,09 vezes maior em animais com invasão vascular, comparado aos demais.

-A presença de invasão vascular e metástase regional foram os fatores que interferiram na sobrevida dos animais, quando feita a análise com pacientes em todos estádios clínicos. Os animais com metástase regional apresentam 2,78 vezes mais chances de óbito comparado aos que não apresentam metástase.

-Os tipos histológicos que demonstraram maior mediana de sobrevida foram o mioepitelioma maligno e carcinoma neuroendócrino, com medianas de sobrevida de 815 dias e 605 dias respectivamente.

-Os tipos histológicos de pior prognóstico quando considerado apenas o estadio inicial (I, II e III), foram o adenomioepitelioma maligno e o carcinoma invasor, com mediana de sobrevida de 365 dias para ambos.

9. CONSIDERAÇÕES FINAIS

O grupo de pesquisa do Laboratório de Patologia Comparada da UFMG vem desenvolvendo ao longo dos últimos 21 anos diversos projetos priorizando a classificação histológica, a abordagem clínica e a padronização de marcadores prognósticos e preditivos em neoplasias mamárias de cadelas. Nesse sentido, há muito tempo já vínhamos observando que os carcinomas sólidos apresentavam características morfológicas diversas, mesmo compartilhando o mesmo tipo de arranjo celular. Foi visto então a necessidade de aprofundarmos nossos estudos acerca desse tipo histológico, que nos trazia poucas informações sobre a sua origem e comportamento biológico.

De acordo com os nossos resultados, acreditamos que o carcinoma sólido é um grupo amplo de neoplasias que compartilham de um arranjo celular comum, porém apresentam características morfológicas distintas, assim como imunofenótipo e comportamento biológico.

Alguns dos tipos histológicos classificados nesse trabalho, como o carcinoma basaloide e o carcinoma papilar sólido, são de fácil distinção morfológica, sem necessidade de diferenciação pela imuno-histoquímica, e podem, portanto, serem identificados na análise histopatológica de rotina. Porém até hoje, apenas nosso grupo tem utilizado na rotina diagnóstica os critérios da OMS.

A sobrevida encontrada nos carcinomas papilares sólidos da mama da cadela, foi diferente daquela descrita na espécie humana. Porém tivemos poucos casos com esse padrão histológico, o que não nos permite afirmar que há diferença de comportamento entre essas espécies.

Uma das limitações do trabalho está relacionada aos dados de acompanhamento clínico, que não foi obtido de 30,4% dos casos. Isso nos limitou quanto a definição real do comportamento biológico dessas neoplasias. Essa dificuldade em se obter os dados de sobrevida acontece com frequência principalmente quando se trabalha com dados de forma retrospectiva.

Como sabemos, o câncer de mama na mulher compreende um grupo heterogêneo influenciado por diversos fatores que vão desde a apresentação morfológica, clínica, molecular e resposta terapêutica. Nessa espécie, a maior parte dos tipos histológicos das neoplasias mamárias já estão

bem estabelecidos, assim como seu imunofenótipo e fatores prognósticos. Nosso grupo de pesquisa tem procurado se aproximar cada vez mais dos critérios de classificação histológica das lesões mamárias da espécie humana. Dessa forma pretendemos contribuir com o diagnóstico e a terapêutica mais especifica e colaborar com o estudo da patologia comparada, visto que a cadela é apontada como um modelo espontâneo do câncer de mama para a espécie humana.

Acreditamos que este seja o início dos estudos acerca das neoplasias mamárias caninas que apresentam arranjo sólido. Nesse sentido, esperamos que em um futuro próximo, o carcinoma sólido deixe de ser um tipo histológico e que o presente trabalho contribua para a elaboração de uma nova classificação. Nosso grupo dará continuidade as pesquisas com os carcinomas com arranjo solido, procurando aumentar a casuística e fazendo a avaliação genotípica e imunofenotípica.

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11. ANEXOS

ANEXO I. Carta de aprovação do CEUA/UFMG – Novembro/2017

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA: 11/2017
Título do projeto: Avaliação morfológica e determinação do fenótipo molecular dos carcinomas sólidos da glândula mamária canina
Finalidade: Pesquisa
Pesquisador responsável: Geovanni Dantas Cassali
Unidade: Instituto de Ciencias Biologicas
Departamento: Departamento de Patologia

Situação atual: Decisão Final - Aprovado

Aprovado na reunião do dia 05/06/2017. Validade: 05/06/2017 à 04/06/2022

Belo Horizonte, 05/06/2017.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/ solicite_ceua/

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ANEXO II. Atividades desenvolvidas no período do doutorado (Março de 2017 a março de 2021)

TRABALHOS RELACIONADOS À TESE

Artigos publicados:
NAKAGAKI, K. Y. R., GONÇALVES, A. B. B., ROCHA, R. M., & CASSALI, G. D. (2019). First description of basaloid carcinoma of the canine mammary gland: case report. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *71*(3), 878-882.

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