ENIO FERREIRA

Análise histomorfológica, imuno-histoquímica e de hibridização cromogênica *in situ* em lesões mamárias proliferativas ductais de cadelas.

Belo Horizonte
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Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais como parte dos requisitos para a obtenção do título de Doutor.

Área de concentração: Patologia Geral

Orientador: Prof. Dr. Geovanni Dantas Cassali

Co-Orientadora: Profa. Dra. Helenice Gobbi

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ATA DA DEFESA DE TESE DE DOUTORADO de **ENIO FERREIRA**, nº de registro 2007652336. Às treze horas do **dia trinta do mês de abril de dois mil e dez**, reuniu-se na Faculdade de Medicina da UFMG, a Comissão Examinadora de tese indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"ANÁLISE HISTOMORFOLÓGICA, IMUNO-HISTOQUIMICA E DE HIBRIDIZAÇÃO CROMOGÊNICA IN SITU EM LESÕES MAMÁRIAS PROLIFERATIVAS DUCTAIS DE CADELAS"**, requisito final para a obtenção do grau de Doutor em Patologia, pelo Programa de Pós-Graduação em Patologia - Área de Concentração em Patologia Geral. Participaram da comissão examinadora, através de viodeoconferência, os professores: Fernando Schmitt e Fátima Gärtner, ambos pertencentes à Universidade do Porto, Portugal. Abrindo a sessão, o Presidente da comissão, Prof. Geovanni Dantas Cassali, após dar a conhecer aos presentes o teor das normas regulamentares do trabalho final passou a palavra ao candidato para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores com a respectiva defesa do candidato. Logo após, a comissão se reuniu sem a presença do candidato e do público para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações:

Prof. Dr. Geovanni Dantas Cassali – Orientador	Instituição: UFMG	Indicação: Aprovado
Profa. Dra. Helenice Gobbi – Coorientadora	Instituição: UFMG	Indicação: Aprovado
Prof. Dr. Fernando Schmitt	Instituição: Universidade do Porto	Indicação: Aprovado
Profa. Dra. Fátima Gärtner	Instituição: Universidade do Porto	Indicação: Aprovado
Profa. Dra. Rogéria Serakides	Instituição: UFMG	Indicação: Aprovado
Dr. Agostinho Pinto Gouveia	Prefeitura Belo Horizonte	Indicação: Aprovado

Pelas indicações, o candidato foi considerado Aprovado.

O resultado final foi comunicado publicamente ao candidato pelo Presidente da comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da comissão examinadora. Belo Horizonte, 30 de abril de 2010.

Prof. Geovanni Dantas Cassali – Orientador
Profa. Helenice Gobbi – Coorientadora
Prof. Fernando Schmitt
Profa. Fátima Gärtner
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DECLARAÇÃO

A Comissão Examinadora abaixo assinada, composta pelos professores doutores: Geovanni Dantas Cassali, Helenice Gobbi, Fernando Schmitt (participação por videoconferência) e Fátima Gärtner (participação por videoconferência), Agostinho Pinto Gouveia, Rogéria Serakides, aprovou a defesa da tese intitulada: **"ANÁLISE HISTOMORFOLÓGICA, IMUNO-HISTOQUIMICA E DE HIBRIDIZAÇÃO CROMOGÊNICA IN SITU EM LESÕES MAMÁRIAS PROLIFERATIVAS DUCTAIS DE CADELAS"**, apresentada pelo doutorando **ENIO FERREIRA** para obtenção do título de Doutor em Patologia, pelo Programa de Pós-Graduação em Patologia - Área de Concentração em Patologia Geral, da Faculdade de Medicina da Universidade Federal de Minas Gerais, realizada em 30 de abril de 2010.

Prof. Geovanni Dantas Cassali
Orientador

Profa. Helenice Gobbi
Coorientadora

Prof. Fernando Schmitt

Profa. Fátima Gärtner

Dr. Agostinho Pinto Gouveia

Profa. Rogéria Serakides

É só isto que desejo fazer: saltar sobre os limites que separam o possível existente do utópico desejado, que ainda não nasceu. Dizer o nome das coisas que não são, para quebrar o feitiço daquelas que são...

Rubem Alves, Estórias de quem gosta de ensinar.

DEDICATÓRIA

Ao meu pai José Ferreira Maciel ,
simplesmente para tentar ser seu reflexo.

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As duas pessoas que acreditaram que eu seria capaz, sem nem eu mesmo saber. Pai e Ellen, obrigado, vocês foram fundamentais.

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Muito obrigado!

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LISTA DE ABREVIATURAS

ACC - Alterações de células colunares

ADH - Hiperplasia ductal atípica

CISH- Hibridização cromogênica *in situ*

DAB - Diaminobenzidina

EGFR - Receptor para fator de crescimento epidérmico

FEA - Atipia epitelial plana

HER1 - Receptor para fator de crescimento epidérmico 1

HER2- Receptor para fator de crescimento epidérmico 2

HLA - Hiperplasia lobular atípica

HLU - Hiperplasia lobular usual (sem atipia)

HMA - Hiperplasia epitelial mista atípica

HMU - Hiperplasia epitelial mista usual (sem atipia)

HUT - Hiperplasia do tipo usual (sem atipia)

ICB - Instituto de Ciências Biológicas

LCC - Lesões de células colunares

RE- Receptor para estrógeno

RP- Receptor para progesterona

UFMG – Universidade Federal de Minas Gerais

RESUMO

A glândula mamária canina, semelhante ao observado na espécie humana, apresenta alterações epiteliais que podem ser divididas em dois grandes grupos: neoplásicas e não neoplásicas. Vários tipos de lesões não neoplásicas são identificadas como alterações precursoras do câncer de mama: hiperplasias ductais e loburares, carcinomas ductais e lobulares *in situ* e, descritas mais recentemente, as alterações de células colunares. As hiperplasias ductais atípicas podem representar lesões precursoras, ou estágios iniciais de desenvolvimento de carcinomas mamários caninos. Com isso, o presente trabalho tem por objetivo caracterizar o aspecto morfológico e analisar a expressão de proteínas relacionadas ao ciclo celular em lesões intraepiteliais não neoplásicas observadas na glândula mamária canina na presença ou ausência de lesões tumorais. A partir da revisão histológica foi observado que essas alterações intraepiteliais estão frequentemente associadas a outras lesões tumorais e são caracterizadas como hiperplasias ductais ou lobulares e lesões de células colunares. Foi identificada uma alta prevalência de lesões não neoplásicas atípicas associadas a carcinomas ductais *in situ* e carcinomas invasores, e a expressão de proteínas envolvidas no ciclo celular apresentam grande variação entre essas lesões e os tumores adjacentes. Dentre as similaridades imuno-histoquímicas observadas entre essas lesões mamárias caninas e humanas destacam-se o aumento da expressão de marcadores de proliferação celular (Ki67) e fraca expressão de receptores hormonais e EGFR. Em contrates com os carcinomas mamários caninos, as lesões hiperplásicas e de células colunares não apresentam alterações da proteína HER2. Foram realizadas técnicas de hibridização cromogênica *in situ* em três casos de carcinomas mamários HER2 positivos, entretanto as lesões neoplásicas e as hiperplasias atípicas associadas não apresentaram amplificação gênica. Nossos resultados demonstram que as lesões epiteliais não neoplásicas mamárias espontâneas são comuns em cães e apresentam um papel importante no processo de transformação neoplásica maligna. De acordo com os nossos conhecimentos, esse é o primeiro estudo a descrever as alterações de células colunares na glândula mamária canina, e essas lesões são morfologicamente e imunofenotípicamente semelhantes as observadas em seres humanos. Conclui-se que a espécie canina poderia representar um modelo adequado em novos estudos sobre cancer, devido as suas similaridades morfológicas e genotípicas com as lesões humanas. Estudos prospectivos avaliando o comportamento molecular poderão contribuir para melhor compreensão desses achados no desenvolvimento do câncer de mama na espécie canina.

Palavras-chave : Glândula mamária, lesões intraepiteliais, cancer, cão.

ABSTRACT

The canine mammary gland bears significant pathological lesions similar to the human breast and epithelial lesions can be divided into two major groups: non-neoplastic and neoplastic. Various precursor breast non-neoplastic lesions have been implicated in cancer development: ductal and lobular hyperplasia, ductal and lobular carcinoma *in situ*, and more recently columnar cell lesions. Recent molecular studies suggest that some canine hyperplasias, particularly those with cytological atypia, represent precursors to, or an early stage in the development of, invasive mammary carcinoma. Thus, the present work aimed to perform objective was to define the pathologic spectrum and analyze the expression of cell cycle related proteins in canine mammary intraepithelial non-neoplastic lesions of mammary specimens of dogs with and without tumor. The histologic review showed that intraepithelial lesions were associated with other mammary tumors in the majority of the cases. These lesions were represented by ductal and lobular hyperplasias and columnar cell alterations. We found a higher prevalence of non-neoplastic atypical lesions with ductal carcinomas *in situ* and invasive carcinomas, and the expression pattern of cell cycle related proteins was very variable from intraepithelial lesions and the adjacent tumors. Immunohistochemical similarities between human and dog intraepithelial lesions also included, increased expression of the proliferation marker Ki-67 and lack expression of hormonal receptors and EGFR. In contrast to canine mammary cancer, analyses of hyperplastic and columnar lesions presented here did not reveal the presence of HER2 protein alterations. Chromogenic *in situ* hybridization was successful in three HER2 positive cases of mammary carcinomas, however all neoplastic and associated atypical hyperplasias were absent of gene amplification. Our findings demonstrate that the spontaneous epithelial non-neoplastic mammary lesions are common in dogs and may play an important role in the process of malignant neoplastic transformation. To our knowledge, our study is the first to describe columnar cells lesions in the mammary glands of female dogs and these lesions are pathologically and immunophenotypically similar to those in human breast. We may conclude that the canine species could be the most adequate model for new studies for cancer, due to the morphological and genotypic similarities of human lesions. Prospective studies evaluating the lesions molecular behavior may contribute to a better understanding of these changes in canine mammary cancer development.

Key-words : Mammary gland, intraepithelial lesions, cancer, dog

1. INTRODUÇÃO

1.1– Modelos histopatológicos de progressão para o câncer de mama humano

O crescimento e a diferenciação do epitélio mamário dependem da ação de fatores de crescimento, de citocinas e, principalmente, da estimulação hormonal (MacDonnell, Norris, 2002; Doisneau-Sixou *et al.*, 2003). Podemos destacar os hormônios sexuais femininos, estrógeno e progesterona, e os hormônios do crescimento, como aqueles de grande significância no estudo da fisiologia mamária normal e de suas alterações de crescimento e diferenciação celular (Ito *et al.*, 1972; Pike *et al.*, 1993; Henderson, Feigelson, 2000).

Diferentes estudos, epidemiológicos, clínicos e moleculares demonstram que o desenvolvimento de neoplasias mamárias está relacionado diretamente a mudanças no comportamento gênico e molecular, comprometendo os mecanismos envolvidos no controle do ciclo celular.

Apesar de ser demonstrado o potencial pré-neoplásico de determinadas proliferações benignas, não existe um critério definido de modelo de progressão, principalmente porque diferentes alterações podem estar relacionadas a diferentes desenvolvimentos de carcinomas mamários (Hartmann *et al.*, 2005). Com isso sugere-se que alterações consideradas intraepitelias ou intraductais não neoplásicas, podem representar, na verdade, diferentes etapas no processo de transformação celular que progridem para o surgimento de carcinomas invasores na mama (Page, 1996).

A característica fundamental das hiperplasias epiteliais mamárias é aumento relativo no número dessas células acima da membrana basal ducto-alveolar. Essas são diferenciadas em lesões mais brandas associadas principalmente ao aumento no número celular sem grandes alterações celulares e nucleares (hiperplasias usuais), até lesões mais severas, onde podemos observar acentuadamente células monomórficas, com alterações principalmente nucleares (hiperplasias atípicas) (Page, 1987).

Na classificação das lesões mamárias humanas são reconhecidos três tipos distintos de hiperplasias epiteliais que apresentam diferentes potenciais de transformação maligna: as hiperplasias lobulares, ductais e apócrinas.

As hiperplasias lobulares são assim denominadas por ocorrerem nas unidades lobulares da mama e estarem associadas principalmente ao carcinoma lobular. Nesse tipo de lesão as células epiteliais possuem o comportamento morfológico bem distinto, com as células apresentando núcleos pequenos, redondos, em sua maioria centralizados e de baixo pleomorfismo, por vezes apresentando um único vacúolo intracitoplasmático (Page, 1987). O risco de surgimento de carcinomas bilaterais a partir dessas lesões, com grande evidência para as lesões atípicas, é demonstrado em diferentes estudos de acompanhamento clínico (Carter *et al.*, 1988; Page *et al.*, 1988; Page *et al.*, 2003). Alterações cromossomais, com perda ou ganhos de sequências gênicas demonstram cada vez mais o potencial pré-maligno dessas lesões hiperplásicas (Lu *et al.*, 1998).

O segundo, e mais comum tipo de hiperplasia, as hiperplasias ductais, referem-se às proliferações caracterizadas por projeções extranumerárias de células epiteliais tanto no interior dos ductos terminais quanto nas formações lobulares. Consideram-se comportamentos celulares atípicos, quando essas organizações hiperplásicas apresentam projeções uniformes com uma delicada monotonia nuclear, dificultando, em alguns casos, a distinção entre uma lesão hiperplásica atípica e um carcinoma ductal *in situ* de baixo grau. Nessas situações a avaliação do número e tamanho da lesão deve ser considerada como critério de distinção dessas lesões, entretanto ambas as lesões são conhecidas como potencialmente malignas, principalmente quando presentes em mulheres com histórico familiar para o câncer de mama (Tavassoli, Norris, 1990; Page, Rogers, 1992). Estudos moleculares demonstram que as hiperplasias ductais atípicas e os carcinomas ductais *in situ* de baixo grau, aparentemente, apresentam semelhantes alterações cromossomais e possuem provavelmente papel similiar no processo de transformação maligna na mama (Sgori, 2010).

O terceiro tipo e mais raro de hiperplasia caracteriza-se por formações papilares ducto-alveolares associadas ao aparecimento de células similares às células apócrinas de glândulas sudorípas e é classificada como hiperplasia apócrina da mama. A relação entre a hiperplasia apócrina e câncer de mama ainda é controversa, pois análises citogenéticas demonstram que esse tipo de hiperplasia pode apresentar-se como uma alteração monoclonal com uma série de mudanças genéticas. Entretanto essas lesões não podem ser consideradas obrigatoriamente lesões precursoras de carcinomas *in situ* ou invasores e o seu significado clínico ainda permanece incerto (Jones *et al.*, 2001; Celis *et al.*, 2007).

Outro padrão de proliferação intraepitelial não neoplásica, as lesões de células colunares (LCC), têm se destacado nos últimos anos em relação ao seu potencial de transfomação maligna. As LCCs estão associadas a presença de microcalcificações no exame mamográfico e presumivelmente representa uma possível etapa para o desenvolvimento de algumas formas de carcinomas *in situ* de baixo grau e carcinoma invasor (Reis-Filho, 2003; Dabbs, 2006).

As alterações de células colunares observadas na mama humana compreendem um grupo de processos caracterizados por dilatações dos ductos terminais organizados em um padrão colunar, contendo uma ou duas camadas celulares com ou sem atipia (Schnitt, 2003). Estudos recentes sugerem a existência de uma relação entre as LCCs com a presença do carcinoma tubular e a coexistência de lesões de células colunares atípicas com o carcinoma ductal *in situ*. (Sahoo, Recant, 2005; Leibl *et al.*, 2007).

Aparentemente o comportamento morfológico das alterações epiteliais mamárias está diretamente relacionado com o aumento do risco de desenvolvimento do câncer de mama. A partir dessa hipótese, o processo de carcinogênese poderia apresentar diferentes etapas de transformação celular: primeiramente ocorreria um estímulo à proliferação epitelial típica (hiperplasia típica), posteriormente esse epitélio se tornaria atípico caracterizado principalmente por alterações em sua constituição gênica (hiperplasia atípica) e só então se transformaria em um carcinoma não invasivo que progrediria assim para a invasão tecidual e formação de metástases em órgãos distantes (Lakhani, 1999).

Nesse modelo de progressão neoplásica podemos observar dois grandes grupos de lesões que são exploradas em estudos distintos: os carcinomas lobulares e ductais invasores. Aparentemente o surgimento de carcinomas lobulares invasores possuem um processo de progressão neoplásica a partir de hiperplasias lobulares atípicas e carcinomas lobulares *in situ*. No entanto, os carcinomas ductais invasores podem sofrer dois tipos de progressão neoplásica: a partir de lesões de células colunares; ou a partir de hiperplasias ductais sem atipia celular ou com atipia celular (Sgroi, 2010).

Os diferentes tipos de lesões intraepiteliais apresentam diferentes riscos de transformação maligna. As hiperplasias epiteliais ductais usuais são caracterizadas como lesões de baixo risco, por aumentarem em torno de 1.5 a 2 vezes a chance de desenvolvimento de carcinomas mamários. Contudo, o surgimento de hiperplasias ductais atípicas, de carcinomas ductais *in*

situ e das alterações de células colunares atípicas aparentemente estão associadas a uma elevação nos riscos de ocorrência de novas neoplasias malignas (Krogerus, 2000; Dabbs, 2006).

Apesar do reconhecimento epidemiológico do potencial maligno das alterações não neoplásicas epiteliais, diferentes estudos se conflitam em inferir sobre a identificação das alterações citogenéticas e moleculares que podem caracterizar o papel dessas lesões epiteliais no processo de progressão neoplásica mamária (Boecker, 2004).

1.2 - Lesões mamárias caninas: modelo no estudo de progressões neoplásicas

Os tumores da glândula mamária de cães têm interesse especial em oncologia devido ao seu surgimento espontâneo e suas similaridades com o câncer humano. Tal fato sustenta a hipótese defendida por diferentes autores, que propõem a utilização da espécie canina como um modelo em oncologia comparada (Schneider, 1970; Peleteiro, 1994; Cassali, 2000; Geraldes et. al, 2000).

A partir de mudanças observadas no comportamento molecular do epitélio mamário canino, sugere-se que lesões intraepiteliais ou intraductais (hiperplasias ductais e carcinomas *in situ*) representam etapas evolutivas no processo de progressão neoplásica maligna (Antuofermo *et al.*, 2007; Jakab *et al.*, 2008; Rao *et al.*, 2009).

As hiperplasias epiteliais mamárias da espécie canina são freqüentemente observadas nas terminações ductais da glândula. Essas lesões podem surgir nos ductos extralobulares, denominadas como hiperplasias ductais, ou se formarem nos ductos intralobulares, denominados como hiperplasias lobulares. Esses tipos de hiperplasias podem aparecer de maneira difusa ou multifocal e serem referidas como papilomatose ou epiteliose. Histologicamente, quando as células são pequenas, com núcleos uniformes, e uma camada organizada de células mioepiteliais delimitam o ducto, denomina-se a lesão atípica, seja ela uma hiperplasia ductal ou uma hiperplasia lobular (Misdorp *et al.*, 1999).

Um terceiro tipo de hiperplasia, pouco freqüente em caninos e observada também em estruturas lobulares, são as adenoses, caracterizadas por aumento no número de ácinos e

dilatação de ductos intralobulares associadas às alterações no tecido epitelial, mioepitelial e fibroso periductal. Essa lesão é acompanhada de fibrose periductal (adenose esclerosante) e é pouco freqüente em caninos e aparentemente não está relacionada ao processo de progressão neoplásica (Misdorp *et al.*, 1999).

São raros os estudos relacionados à prevalência, sítios mamários de localização e relação entre o surgimento de hiperplasias ductais e a ocorrência simultânea de neoplasias mamárias caninas. Aparentemente, essas lesões são representadas por proliferações difusas, presentes em maior frequência nas mamas inguinais e abdominais, surgindo em animais entre 7 a 10 anos de idade, principalmente em animais que fazem o uso de hormônios sintéticos a base de progestágenos (Cameron, Faulkin, 1971; Nelson, *et al.*, 1973; Warner, 1976).

Progestágenos podem agir diretamente sobre as células epiteliais mamárias e estimular sua proliferação ou atuar indiretamente ao aumentar os níveis plasmáticos do hormônio do crescimento por estimulação adeno-hipófisária (Concannon *et al.*, 1980; Ishikawa *et al.*, 2000; Marinelli *et al.*, 2004; Perry *et al.*, 2008). A maior ativação de receptores para hormônios do crescimento associado à maior síntese desse hormônio é considerada um fator crucial para o surgimento de hiperplasias e neoplasias mamárias (Rehm *et al.*, 2007).

Resultados apresentados por Antuofermo e colaboradores, em 2007, e Mouser e colaboradores, em 2010, mostram que as hiperplasias ductais e os carcinomas *in situ* apresentam características morfológicas e moleculares semelhantes às observadas na espécie humana. Essas lesões estão frequentemente associadas a carcinomas mamários invasores e apresentam uma correlação positiva com esses tumores em relação à perda na expressão de receptores hormonais e no aumento no índice proliferativo celular. A partir desses resultados, os autores sugerem a utilização da espécie canina como um modelo animal comparativo no estudo de lesões mamárias não invasivas associadas ao processo de progressão para o câncer de mama (Antuofermo *et al.*, 2007; Mouser *et al.*, 2010).

A melhor compreensão dessas alterações hiperplásicas, a partir da caracterização de atipia celular e dos comportamentos moleculares que essas lesões apresentam, pode auxiliar em estudos sobre carcinogênese, progressão e tratamento precoce de neoplasias malignas mamárias.

1.3 - Estudos moleculares em lesões neoplásicas e pré-neoplásicas mamárias: imuno-histoquímica e hibridização *in situ*

A utilização de anticorpos, conhecidos como marcadores moleculares, capazes de identificar determinadas proteínas celulares envolvidas no ciclo celular, pode auxiliar no esclarecimento das alterações genômicas observadas durante o processo de carcinogênese mamária (Peters *et. al.*, 2004). Mesmo com a indisponibilidade da maioria dos anticorpos monoclonais caninos para imuno-histoquímica, é demonstrada a reatividade de anticorpos monoclonais humanos para diversos antígenos de mama da espécie canina (Cassali, 2000).

Dentre os marcadores moleculares estudados no processo de carcinogênese destaca-se o estrógeno, mais precisamente o seu receptor celular (RE), devido ao seu papel na regulação do crescimento e da diferenciação do epitélio mamário normal. A identificação do receptor estrógeno tornou-se foco principal em vários estudos epidemiológicos de fatores de ricos associados ao câncer de mama humano e canino (Ciocca *et al.*, 1997; Sutherland *et al.*, 1998).

Embora seja relatada em tumores mamários humanos e caninos uma elevada expressão de receptores hormonais, existem estudos comparativos entre lesões intraepiteliais neoplásicas e não neoplásicas que descrevem uma diminuição na expressão imuno-histoquímica para receptores de estrógeno no processo de transformação neoplásica (Inaba *et al.*, 1984; Antuofermo *et al.*, 2007; Tang *et al.*, 2006). A menor expressão de receptores para estrógeno em tumores mamários em cães está relacionado à aquisição de características celulares relacionadas à transformação maligna (de Las Mulas *et al.*, 2005).

A presença de receptores hormonais está associada a um maior potencial mitogênico e indução à proliferação celular por ativação de ciclinas no epitélio mamário (Yang *et al.*, 2006). Entretanto, a ausência de expressão de receptores de estrógeno pode ser observada em processos proliferativos na mama, sugerindo que outros mecanismos de controle de proliferação celular podem estar envolvidos no surgimento de hiperplasias mamárias. Dentre as hipóteses propostas, relata-se a ocorrência de uma sinalização parácrina entre células epiteliais positivas e negativas para receptores de estrógeno, sugerindo que a proliferação celular mediada pela regulação hormonal pode ocorrer independente da presença de receptores para estrógeno (Anderson, Clarke, 2004; Lee, *et al.*, 2006).

A ativação de receptores hormonais, além de promover a regulação da expressão de ciclinas, está relacionada ao aumento da proliferação e estímulo a migração celular, em lesões epiteliais mamárias, por regulação da expressão de moléculas de adesão (E-caderina, P-caderina) (Helguero *et al.*, 2008). Além da possibilidade de utilização da análise da E-caderina no estudo diferencial de lesões lobulares e ductais mamárias, é descrito que, por essa proteína apresentar perda de expressão ou funcionalidade em tumores mamários invasivos, provavelmente está associada ao processo de progressão neoplásica (Bratthauer *et al.*, 2002; Knudsen, Wheelock, 2005; Suciu *et al.*, 2008).

A expressão de E-caderina em lesões mamárias caninas comprova o seu papel no auxílio diagnóstico e prognóstico do processo de progressão tumoral. Observa-se uma perda de expressão dessa proteína ao comparar proliferações invasivas e não invasivas e um menor tempo de sobrevida em cães portadores de tumores com baixa expressão para essa proteína (Rodo, Malicka, 2008; Gama *et al.*, 2008). A utilização desse marcador no estudo comparativo de lesões proliferativas pré-neoplásicas pode fornecer informações importantes sobre o caráter evolutivo das hiperplasias mamárias.

Além do estudo do comportamento molecular de lesões pré-malignas, a avaliação da atividade proliferativa celular por meio de marcadores imuno-histoquímicos é estabelecida como importante parâmetro prognóstico em lesões mamárias. Avaliações da expressão de ciclina D1 e do índice proliferativo por quantificação de regiões organizadoras de nucléolo (AgNor`s) demonstram um alto potencial de transformação neoplásica em lesões epiteliais hiperplásicas da mama em cadelas. Entretanto, dentre os marcadores de proliferação um dos mais utilizados é o que identifica a proteína Ki-67, presente em todas as fases do ciclo celular, exceto G0 (Shoker *et al.*, 1999; Sfacteria *et al.*, 2003).

Estudos em medicina veterinária revelam que a imunomarcação mais elevada para Ki67 é observada em tumores mamários malignos, e está correlacionada com a menor marcação para o receptor celular de progesterona nesses tumores, e com a menor sobrevida de fêmeas dessa espécie (Peña *et al.*, 1998; Cassali, 2000; Geraldes, 2000). São relatadas similaridades entre as hiperplasias e o tecido normal quando se compara o índice proliferativo e a presença de marcadores celulares para apoptose (Funakoshi *et al.*, 2000; Wakshlag *et al.*, 2006).

O aumento da expressão desse marcador em diferentes tipos de lesões intraepiteliais aparentemente está relacionado com o aumento do grau histológico da lesão, sendo significativamente elevado em hiperplasias atípicas e carcinomas *in situ* de auto grau (Antuofermo *et al.*, 2007; Mouser *et al.*, 2010). Demonstra-se com isso que a avaliação da expressão de Ki67 pode servir como um parâmetro adequado quando se pretende avaliar o caráter prognóstico e evolutivo de alterações epiteliais não invasoras na mama.

Avaliações prognósticas em tumores mamários indicam uma relação importante entre a expressão de receptores estrogênicos, marcadores de proliferação celular (Ki67) e a expressão de receptores para fator de crescimento epidérmico (HER1; HER2) (Colleoni *et al.*, 2008; Yu *et al.*, 2009). Carcinomas mamários humanos que apresentam elevada expressão para Ki67 e perda de expressão para receptores de éstrogeno geralmente apresentam superexpressão para HER2 (Ariga *et al.*, 2005).

A proteína HER2 é codificada pelo protooncogene cerbB-2, localizado no cromossoma 17 na espécie humana. Dentre as mudanças no comportamento gênico dos tumores, a amplificação do gene HER-2 é descrita como uma importante alteração presente no câncer de mama humano, ocorrendo em aproximadamente 30% dos carcinomas ductais invasivos com correlação positiva com outros fatores prognósticos estabelecidos para o câncer de mama (Slamon *et al.*, 1987; Toikkanen *et al.*, 1992).

Avaliações citogenéticas em tumores mamários caninos revelam que a região cromossomal 1q13.1, responsável pela transcrição da proteína HER2, também é muito afetada por aberrações cromossomais (Murua *et al.*, 2001). Estudos imuno-histoquímicos demonstram que a maior expressão dessa proteína possui correlação positiva com o diagnóstico de malignidade em cães, mas não com a presença de invasão local (Dultra *et al.*, 2004; Gama *et al.*, 2008).

A análise da amplificação gênica do cromossoma 17, responsável pela expressão do gene cerbB-2, têm permitido esclarecer os resultados observados em estudos imuno-histoquímicos de tumores mamários humanos (Dandachi *et al*, 2002; Zhao *et al.*, 2002; Madrid & Lo, 2004). Essa amplificação cromossômica pode ser detectada em tecidos fixados em formol e incluídos em parafina a partir da hibridização de uma sonda centromérica com revelação por um

cromógeno, técnica conhecida como "chromogenic *in situ* hybridization" ou CISH (Zymed, 2005).

Estudos de hibridização cromogênica *in situ* (CISH), em diferentes displasias e neoplasias mamárias, demonstram não haver relação entre a superexpressão da proteína HER-2 e a amplificação gênica para HER2 no câncer de mama canino (de Las Mulas *et al.*, 2003). Antuofermo *et al.* (2007), corroborado por Mouser *et al.* (2010) relatam uma ausência de expressão imuno-histoquímica para HER2 em hiperplasias mamárias caninas. Entretanto, o significado do comportamento molecular dessa proteína em relação ao processo de progressão neoplásica ainda permanece pouco claro visto que em alguns casos é observada expressão elevada dessa proteína na glândula mamária normal canina. Evidências recentes sugerem que o aumento da transcrição da proteína HER-2 pode ocorrer por ativação de diferentes fatores de transcrição nuclear (proteínas AP-2 e YY1) (Allouche *et al.*, 2008).

2 – JUSTIFICATIVA

A partir do exposto, para estudos comparativos com a espécie humana, as neoplasias mamárias espontâneas caninas podem ser consideradas, modelos animais mais adequados que outros modelos experimentais. Entretanto, os diferentes critérios histológicos utilizados na classificação, os raros trabalhos relacionados à sobrevida e prognósticos dos animais acometidos por tumores mamários, a falta de dados epidemiológicos e os escassos estudos de biologia molecular são fatores limitantes na comparação entre os tumores dessas duas espécies.

Aparentemente os estudos de lesões hiperplásicas ductais mamárias caninas mostram-se promissores quando se objetiva identificar quais proliferações mamárias caninas estão envolvidas no processo de transformação maligna. Entretanto, a tentativa de se estabelecer os fatores relacionados ao processo de progressão neoplásica na mama canina deve ser acompanhada de uma caracterização rigorosa das lesões mamárias não invasivas. Inicialmente a proposta de caracterização morfológica dessas lesões deve ser bem estabelecida para melhor identificação dos aspectos moleculares presentes em cada tipo de lesão a ser estudada - foco do trabalho a ser apresentado.

3. HIPÓTESE

Em semelhança à espécie humana, as lesões mamárias intraepiteliais não neoplásicas da cadela podem apresentar características oncogênicas indicativas de transformação neoplásica.

4. OBJETIVOS

4.1 - Objetivo geral

Caracterizar morfologicamente e identificar por utilização de marcadores imuno-histoquímicos e da técnica de hibridização *in situ* com revelação por cromógeno (CISH) a relação entre o desenvolvimento de lesões intraepiteliais e o potencial de transformação maligna dos componentes epiteliais mamários na cadela.

4.2 - Objetivos específicos

I) Revisão histológica das lesões mamárias intraepiteliais não neoplásicas da cadela de acordo com a classificação veterinária (Misdorp *et al.*, 1999) e humana (Tavassoli *et al.* , 2003).

II) Descrição dos tipos mais freqüentes de alterações epiteliais hiperplásicas ou em transformação carcinomatosa *in situ* em glândulas mamárias caninas e de suas associações com outras lesões mamárias neoplásicas.

III) Caracterização da expressão imuno-histoquímica dos marcadores tumorais RE, RP, Ki-67, HER-2, EGFR, citoqueratina 34βe-12 e E-caderina nos diferentes tipos de lesões mamárias intraepiteliais não neoplásicas da cadela, em carcinomas *in situ* e nos carcinomas invasivos associados.

IV) Identificação do comportamento do gene HER-2, por meio da técnica de CISH, e correlação com a expressão da proteína HER-2 nos diferentes tipos de lesões intraepiteliais mamárias caninas e nos carcinomas invasivos associados a essas lesões.

5. MATERIAL E MÉTODOS

5.1 - Espécimes mamários

As lesões mamárias foram obtidas de cadelas de várias raças, puras ou mestiças, que sofreram extirpação cirúrgica no Hospital Veterinário da Escola de Veterinária da UFMG, ou de material dos arquivos do setor de Patologia do Departamento de Clínica e Cirurgias Veterinárias da mesma Escola e do Laboratório de Patologia Comparada – ICB/UFMG.

Todos os procedimentos experimentais foram executados segundo as recomendações e a aprovação do Comitê de Ética em experimentação animal da Universidade Federal de Minas Gerais (CETEA/UFMG), sob o número de protocolo 192/2006 (ANEXO I).

5.2 – Histopatologia

Foram obtidas secções histológicas de 4 μm da glândula mamária, de material fixados por 24 horas em formol neutro e tamponado a 10%, processados pela técnica rotineira de inclusão em parafina e corados pelas técnicas da hematoxilina-eosina. Para classificação morfológica das lesões mamárias foram estabelecidas as descrições propostas pela classificação veterinária (Misdorp *et al.*, 1999) e humana (Tavassoli *et al.*, 2003). Após a revisão dos casos, foram selecionadas as amostras que apresentaram proliferações intraepiteliais mamárias puras ou associadas a outras lesões neoplásicas epiteliais invasivas e não invasivas. O diagnóstico histológico foi feito com base no protocolo proposto por Ferreira e colaboradores (2003) (ANEXO II).

5.3 -Imuno-histoquímica

Após a revisão dos casos, secções histológicas de 4 μm das amostras selecionadas foram coradas pela técnica de imuno-histoquímica.

No procedimento imuno-histoquímico foi realizada a técnica de reação biotina-peroxidase com identificação a partir de anticorpo secundário polimerizado (ADVANCE HRP – ready to

use – DakoCytomation). A recuperação antigênica foi realizada em calor úmido (98oC), com solução de tampão citrato pH 6,0 (DakoCytomation Target Retrieval Solution) durante 20 minutos. Para realização de bloqueio da peroxidase endógena as lâminas foram incubadas em solução de H_20_2 3% em álcool metílico. Os reagentes foram aplicados pela técnica manual, sendo o tempo de incubação do anticorpo primário de 1 hora e os demais reagentes de 30 minutos, com exceção do cromógeno DAB (DAB substrate system, Dakocytomation), de cinco minutos. Como controles positivos foram usadas amostras de glândula mamária de cadela previamente testadas e os controles negativos foram obtidos por substituição do anticorpo primário pelo soro normal.

Os anticorpos primários utilizados estão descritos na tabela 1, com suas respectivas fontes e diluições. A imunomarcação para RE, RP, P53, citoqueratina 34βe-12 e E-caderina foi avaliada semi-quantitativamente, sendo determinado cinco categorias para as células com alterações proliferativas: negativo (-), quando <5% das células foram positivas; positivo (+), quando 5% a 25% das células foram coradas; positivo (++), quando 25% a 50% das células foram coradas; positivo (+++), quando 50% a 75% das células foram coradas; difusamente positivo (++++), quando >75% das células foram coradas. O índice proliferativo foi calculado por meio da contagem de células imuno-marcadas para a proteína Ki-67 em um total de 500 epiteliais com alterações proliferativas. Para determinação da expressão de HER-2 e EGFR seguiu-se a determinação da Sociedade Americana de Oncologia Clínica/Colégio Americano de Patologia (ASCO/CAP), sendo considerado imuno-positivas as lesões que apresentassem marcação membranar forte e contínua em um número superior a 30% das células epiteliais com alteração de proliferação (Wolff *et al.* 2007).

5.4 –Hibridização *in situ* com revelação cromogênica (CISH)

Para a realização da CISH, foi utilizado o *kit* para detecção CISH SPOT-Light Chromogenic ISH (Zymed Laboratories Inc). Secções histológicas de 4 μm obtidas dos tecidos hiperplásicos montadas em lâminas silanizadas foram hidradas e pré-tratadas de acordo com os protocolos fornecidos pelo kit. Em seqüência, as lâminas foram incubadas com a sonda do gene HER-2 (SPOT-Light HER-2 Probe), no Hybridizer Dako por 5 minutos à temperatura de 900 C e 10 horas (overnight) à temperatura de 370 C. A sonda a ser hibridizada foi detectada utilizando o sistema de detecção CISH Polymer Detection Kit II e os tecidos foram contra-

corados com Hematoxilina de Mayers por 10 segundos. Como controles positivos foram usadas amostras de mama humana previamente testada.

A análise e interpretação da hibridização foi de acordo com recomendação fornecida pela Zymed junto ao protocolo do kit CISH SPOT-Light Chromogenic ISH (Zymed Laboratories Inc). Para a análise da hibridização foram contadas 30 células neoplásicas em microscópio óptico em objetiva de 40x. A interpretação seguiu o proposto no Quadro 1.

Tabela 1 - Anticorpos primários, fonte e diluições padronizadas no estudo imuno-histoquímico.

Anticorpo (Clone)	Fonte	Diluição*
RE (LH2)	Novocastra	1:20
RP (HPRA2)	Neomarkers	1:20
Ki-67 (MIB-1)	Dakocytomation	1:25
Citoqueratina 1, 5, 10 e 14 (34βe12)	Dakocytomation	1:40
E-caderina (4A2C77)	Zymed	1: 100
HER-2 (A0485)	Dakocytomation	1:40
EGFR	Dakocytomation	1:100
P53 (CM1)	Covance	1:50

*Diluição padronizada no Laboratório de Patologia Comparada

Quadro 1 – Determinação do estado do cromossoma 17 e HER-2 por meio da detecção pelo CISH.

Sem amplificação	1 a 4 cópias do gene presentes em cada núcleo em mais de 50% das células neoplásicas.
Amplificação baixa	5 a 10 cópias, ou pequenos aglomerados do gene presentes em cada núcleo em mais de 50% das células neoplásicas
Amplificação alta	mais de 10 cópias ou aglomerados do gene presentes em cada núcleo em mais de 50% da células neoplásicas

6. RESULTADOS

Os resultados serão apresentados sob a forma de três artigos científicos elaborados (publicados e submetidos) durante o período de doutoramento.

ARTIGO 1

Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypic analysis

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Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypic analysis

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Abstract

Background: It has been suggested that columnar cell lesions indicate an alteration of the human mammary gland involved in the development of breast cancer. They have not previously been described in canine mammary gland. The aim of this paper is describe the morphologic spectrum of columnar cell lesions in canine mammary gland specimens and their association with other breast lesions.

Methods: A total of 126 lesions were subjected to a comprehensive morphological review based upon the human breast classification system for columnar cell lesions. The presence of preinvasive (epithelial hyperplasia and in situ carcinoma) and invasive lesions was determined and immunophenotypic analysis (estrogen receptor (ER), progesterone receptor (PgR), high molecular weight cytokeratin (34βE-12), E-cadherin, Ki-67, HER-2 and P53) was perfomed.

Results: Columnar cell lesions were identified in 67 (53.1%) of the 126 canine mammary glands with intraepithelial alterations. They were observed in the terminal duct lobular units and characterized at dilated acini may be lined by several layers of columnar epithelial cells with elongated nuclei. Of the columnar cell lesions identified, 41 (61.2%) were without and 26 (38.8%) with atypia. Association with ductal hyperplasia was observed in 45/67 (67.1%). Sixty (89.5%) of the columnar cell lesions coexisted with neoplastic lesions (20 in situ carcinomas, 19 invasive carcinomas and 21 benign tumors). The columnar cells were ER, PgR and E-cadherin positive but negative for cytokeratin 34βE-12, HER-2 and P53. The proliferation rate as measured by Ki-67 appeared higher in the lesions analyzed than in normal TDLUs.

Conclusions: Columnar cell lesions in canine mammary gland are pathologically and immunophenotypically similar to those in human breast. This may suggest that dogs are a suitable model for the comparative study of noninvasive breast lesions.

Background

The development of human breast cancer is believed to be a complex multistep process originating in terminal duct lobular units (TDLUs) and progressing towards invasive cancer. Various precursor breast lesions have been implicated in cancer development: atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), lobular carcinoma in situ (LCIS), ductal carcinoma in situ (DCIS) and more recently columnar cell lesions (CCLs) [1-2].

Columnar cell lesions (CCL) of the human breast comprise a group of conditions characterized by varying degrees of acinar dilation in the TDLUs, lined by several layers of columnar epithelial cells with uniform, ovoid nuclei oriented perpendicular to the basement membrane. The number of cellular layers enable CCLs to be divided into two broad categories: columnar cell change (CCC) [1-2 cell layers] or columnar cell hyperplasia (CCH) [>2 cell layers]. CCC and CCH with cytological atypia are further subclassified as flat epithelial atypia (FEA) [3-5].

Recent observational studies and emerging genetic evidence suggest that some CCLs, particularly those with low-grade/monomorphic-type cytological atypia, represent precursors to, or an early stage in the development of, low-grade ductal carcinoma in situ (DCIS) and invasive carcinoma [1, 6].

The canine mammary gland bears significant pathological lesions similar to the human breast [7,8]. Breast lesions in dogs show cellular changes involved in the progression to invasive carcinoma. They are known as atypical hyperplasia and carcinoma in situ [9,10,11]. These descriptions may suggest that dogs are a promising model animal for comparative oncology. Therefore, a clearer account of the alterations in canine mammary cancer will help to better understand the key steps in the formation of human tumors.

In this paper we describe the presence of CCLs in canine mammary gland specimens, their association with other breast lesions and immunohistochemical findings in a series of specimens.

Methods

Specimen selection

Specimens from one hundred and twenty-six consecutive cases of canine mammary gland, with previous diagnosis of epithelial lesions, were selected from the archives of the Laboratory of Comparative Pathology of the Biological Science Institute of the Federal University of Minas Gerais. The mammary gland samples were obtained after clinical diagnosis of mammary tumor and surgical removal of the lesion. Hematoxylin and eosin–stained sections were reviewed to search for columnar cell and associated lesions. The ages of the animals at the time of surgery ranged from 3 to 16 years (mean 9.8 years ± 2.2).

One human pathologist (HG) and two veterinary pathologists (EF and GDC) individually reviewed and classified the CCL in terms of the Schnitt and Vincent-Salomon classification. Lesions were divided into two categories according to their distinguishing morphological features: columnar cell change (CCC) and columnar cell hyperplasia (CCH), including subclassifications of these according to the absence or presence of cytological atypia (Table 1) [4, 5]. A consensus classification was achieved for each case by discussion and observation of each individual lesion on a multihead microscope. The canine mammary neoplasias and epithelial hyperplasias were classified according to veterinary nomenclature [12]. The association of CCL with malignant and benign lesions was analyzed using Fisher's exact test with significance at $P < 0.05$.

Immunohistochemistry

Considering the small size of the lesions, the immunohistochemical analysis were performed only on cases with enough material. Paraffin blocks were selected from six cases containg columnar cell lesions. Consecutive 5 µm thick sections were obtained and mounted on silanated slides for immunohistochemical study. Sections were stained for rabbit polyclonal antibodies: HER-2 (c-erbB-2; Dako; dilution: 1:40), P53 (clone CM1; Covance; diluition: 1:80); and mouse monoclonal antibodies: E-cadherin (clone 4A2C7; Zymed; dilution: 1:100), ER-LH2 (clone CC4-5; Novocastra; dilution: 1:25), PgR (clone hPRa2; Neomarkers; dilution: 1:20), Ki-67 (clone Mib-1; Dako; dilution: 1:25), cytokeratins 1, 5, 10 and 14 (clone 34βE-12; Dako; dilution: 1:40). Heat-induced epitope retrieval (20 min) using Dako antigen retrieval solution, pH 6.0 (Dako), was previously performed in a water bath. The slides were then cooled to room temperature for 20 min in the antigen retrieval buffer. The sections were incubated at room temperature in 3% (vol/vol) H_2O_2 for 15 min, in primary antibodies for 16 h,

in reagent contained anti-mouse and anti-rabbit secondary antibodies (Biotinylated Goat Anti-polyvalent, Laboratory Vision) for 15 min and streptavidin peroxidase (UltraVision Large Volume Detection System, HRP, Laboratory Vision) for 15 min. Between incubations, the slides were washed for 2 x 5 min in phosphate-buffered saline containing 1% (vol/vol) Tween 20. Immunoreactivity was visualized by incubating the slides for 10 min with diaminobenzidine (DAB Substrate System; Laboratory Vision). The slides were then counterstained with Harris hematoxylin. Positive and negative control slides were included in each batch. As a positive control we used human breast cancer tissue known to express of the antibodies. Negative controls were assessed using normal serum (Ultra V Block, Laboratory Vision) as the primary antibody.

Staining for ER, PgR, P53, CK34βE-12 and E-cadherin was evaluated semi-quantitatively and scored into five categories: negative (-), <5% of cells stained; positive (+), 5% to 25% of cells stained; positive (++), 25% to 50% of cells stained; positive (+++), 50% to 75% of cells stained; diffusely positive (++++), >75% of cells stained [13]. The proliferative index was calculated by counting the positive nuclei for Ki-67 staining in a total of 500 columnar cells from each lesion. HER-2 expression was defined as epithelial cell membrane staining and scored according to the American Society of Clinical Oncology, College of American Pathologists [14].

All procedures were performed under the guidelines and with the approval of the Ethics Committee in Animal Experimentation (CETEA/UFMG), protocol 192/2006.

Results

CCLs were identified in 67 (53.1%) canine mammary glands from the 126 specimens studied. CCL without atypia were identified in 41/67 (61.1%) canine mammary specimens. The CCC, 39 cases, were characterized by dilated acini lined with a single layer of columnar epithelial cells with elongated nuclei, a small amount of cytoplasm and apical cytoplasm frequently containing snouts and intraluminal secretions (Figure 1). Only two specimens the columnar lesions showed more than two cell layers and had prominent apical cytoplasmic snouts; these were classified as columnar cell hyperplasia without atypia (CCH) (Figure 2).

FEA was found in 26/67 (38.8%) (24 cases of CCCs and 2 cases of CCHs) canine mammary lesions. In these cases the alterations were characterized by the presence of columnar epithelial cells with round to ovoid and/or hyperchromatic nuclei that were not perpendicularly

oriented to the basement membrane, with a slight increase in the nuclear/cytoplasmic ratio (Figure 3). Focal micropapillae and tufting of cells were seen.

The intraluminal microcalcifications were detected in columnar cell changes (6 cases without atypia and 6 cases with atypia) and in columnar cell hyperplasia (only 1 case without atypia).

Associated lesions were represented only by ductal and lobular hyperplasias and epithelial neoplastic lesions. Sixty cases of the CCLs (89.5%) showed coexisting neoplastic lesions (20 ductal carcinomas *in situ*, 19 invasive carcinomas and 21 benign tumors). We found a higher prevalence of FEA with ductal carcinomas *in situ* (13 cases; 50%) than invasive carcinomas (5 cases; 19%) and benign tumors (3 cases; 11.5%). CCCs were detected in association with 18 benign tumors (46.1%), 7 ductal carcinomas *in situ* (17.9%) and 12 invasive carcinomas (30.7%) ($P<0.05$). Two cases of CCH proved to be associated with invasive carcinoma (Table 2).

Ductal hyperplasia was associated with CCLs in 45/67 (67.1%) cases (23 without atypia and 22 with atypia) (Figure 1D). Two cases of CCHs was associated with ductal hyperplasia. Only one atypical lobular hyperplasia was detected. This lesion was associated with columnar cell hyperplasia without atypia and in situ carcinoma. FEA was associated with ductal hyperplasias with atypia in 12 out 17 cases and CCC was more frequently associated with hyperplasias without atypia: 17 out 26 cases ($P<0.05$) (Table 3).

The luminal epithelial cells with columnar change (five CCC and one CCH) showed a strong uniform nuclear immunopositivity for ER and PR antibodies in 50% and around 100% of cells respectively in all six cases studied (Figure 4A and 4B). Strong E-cadherin expression was detected in 5 out of 6 CCLs analyzed with a staining pattern similar to the adjacent normal TDLU. Within these specimens, three cases exhibited strong E-cadherin immunoreactivity in all cells and two exhibited strong immunoreactivity but with focal areas of reduced or absent immunostaining, typically affecting small isolated tufts of cells (Figure 4C).

The proliferation rate as measured by Ki-67 appeared higher in CCLs (mean 4.7%) than normal TDLU. Ki-67-positive cells were generally present but in low numbers in normal internal control tissue epithelium (<1% positive cells) (Figure 4D).

The CCLs were negative for cytokeratin 34βE-12, P53 and HER-2 in all six cases studied. Interestingly, P53 and HER-2 negative results were similar to those obtained with the

associated benign and malignant tumors (two benign mixed tumors, one duct papilloma, one solid carcinoma and two ductal in situ carcinoma).

Discussion

Columnar cell alterations of the canine mammary gland have been documented during microscopic analysis of breast tissues. A common component of most hyperplastic ductal lesions was columnar alteration (also referred to as columnar cell metaplasia) [12]. However, histopathological criteria that characterize columnar lesions in dogs and their malignant potential when detected as the sole histopathological abnormality following breast biopsy are undocumented.

The morphological appearance of the canine CCLs was very similar to the human breast. Hyperplastic breast lesions, such as ductal hyperplasias without and with atypia, have previously been described in the canine mammary gland [12]. To our knowledge, our study is the first to describe CCLs in the mammary glands of female dogs. In the present study we found CCLs in 53.1% of consecutive canine breast specimens. Lesions ranged from CCCs without atypia (61.1%) to FEA (38.8%).

Observational studies have revealed a relationship between CCLs and tubular carcinoma [15, 16]. Columnar cell lesions, in particular those with nuclear atypia, may also co-exist with lobular in situ neoplasia. Flat epithelial atypia is associated with established DCIS more frequently than by chance [17, 18].

Microcalcifications were also frequently found in association with canine CCLs, similar to human lesions. Although CCLs have long been recognized in the human breast under different names, their importance increased after more were diagnosed in mammographically detected lesions owing to microcalcifications [16, 18]. Although mammography is not routinely used in canine species we supposed that such microcalcifications would have a radiological appearance similar to that in the human breast.

Myoepithelial/basal and some epithelial cells in human CCLs are positive for CK5/6 [19]. Though columnar cell lesions in human breast are usually negative for HER-2／neu, p53 and basal CK5／6 and CK14. Similar expression was found in all six cases studied, supporting the use of breast tumor biomarkers in this model. The immunohistochemical similarities between human and dog columnar cells also included, increased epithelial expression of the

proliferation marker Ki-67/MIB1 and strong expression of hormonal receptors (ER and PR) and E-cadherin [13, 20, 21].

The canine species could be the most adequate model for new studies for cancer, due to the morphological and genotypic similarities of human lesions [20, 22, 23]. Spontaneous epithelial mammary lesions are common in dogs well before the age of onset of palpable mammary tumors [9, 7]. Thus, spontaneous tumours of canine mammary glands have been proposed as comparative models for the study of human breast cancer, since these lesions share epidemiological, clinical, behavioural and antigenic features [23, 24, 25].

Conclusions

Columnar cell lesions in canine mammary gland are pathologically and immunophenotypically similar to those in human breast. This may suggest that dogs are a suitable model for the comparative study of noninvasive breast lesions. Additional studies are needed to analyze the frequency of columnar cells lesions and their relationship to in situ carcinomas in canine mammary glands to provide a model for testing treatment modalities for mammary lesions and ultimately for clarifying patient management.

Abbreviations

ADH: Atypical Ductal Hyperplasia; ALH: Atypical Lobular Hyperplasia; CCLs: Columnar Cell Lesions; CCC: Columnar Cell Change; CCH: Columnar Cell Hyperplasia; DCIS: Ductal Carcinoma In Situ; DH: Ductal Hyperplasia without atypia; ER: Estrogen Receptor; FEA: Flat Epithelial Atypia; LCIS: Lobular Carcinoma In Situ; PgR : Progesterone Receptor
TDLUs: Terminal Duct Lobular Units

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EF conceived the study, participated in the immunoassays, performed the statistical analysis and drafted the manuscript. ***HG*** participated in the study design and helped to draft the manuscript. ***BS*** carried out the immunoassays and participated in the design of the study. ***GDC*** participated in design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Histologic features of the different categories of columnar cell lesions in canine mammary gland.

Columnar Cell Change without atypia (CCC)	*Columnar Cell Hyperplasia (CCH)*	*Flat Epithelial Atypia (FEA)*	*Flat Epithelial Atypia (FEA)*
		CCC with atypia	*CCH with atypia*
One to two columnar cell layers with uniform ovoid to elongated nuclei; nucleoli absent or inconspicuous.	Cellular stratification more than two columnar cell layers with uniform ovoid to elongated nuclei; nucleoli absent or inconspicuous.	One to two columnar cell layers with complex architectural patterns present; Mild to moderate cytologic atypia present (usually low-grade): round-to-ovoid, mildly pleomorphic and hyperchromatic nuclei, with inconspicuous nucleoli.	Cellular stratification more than two columnar cell layers with complex architectural patterns present; Mild to moderate cytologic atypia present (usually low-grade): round-to-ovoid, mildly pleomorphic and hyperchromatic nuclei, with inconspicuous nucleoli.

Classification categories are based on an expanded version of those describes by Schitt and Vincent- Salomon [5].

Table 2: Types of canine mammary columnar cell lesions and associated neoplastic alterations.

Tumor types[(**)]	*CCC(%)*	*CCH(%)*	*FEA(%)*	*Total(%)*
invasive carcinomas	**12 (30.7)**		**5 (19.2)**	**19 (28.4)**
carcinoma in benign tumor	*6 (15.3)*	*2 (100)*	*3 (11.5)*	
solid carcinoma	*2 (5.1)*	-	*2 (7.2)*	
tubulopapillary carcinoma	*4 (10.3)*	-	-	
ductal carcinoma *in situ*	**7 (17.9)**	**-**	**13 (50.0)** [(*)]	**20 (29.8)**
benign tumors	**18 (46.1)** [(*)]		**3 (11.5)**	**21 (31.4)**
simple adenoma	*3 (7.6)*	-	-	
duct papilloma	*7 (17.9)*	-	*1 (3.8)*	
benign mixed tumor	*8 (20.6)*	-	*2 (7.7)*	
without tumor	**2 (5.1)**	**-**	**5 (19.2)**	**7 (10.4)**
Total	39 (100)	2 (100)	26 (100)	**67 (100)**

[(*)]Flat epithelial atypia presence is often associated with in situ carcinomas and columnar cell lesion without atypia are more frequent in benign tumors (P<0,05).
[(**)]Misdorp,1999.*International Histological Classification of Tumors of Domestic Animals*.
CCC: columnar cell change without atypia; CCH: columnar cell hyperplasia without atypia; FEA: flat epithelial atypia

Table 3: Types of canine mammary columnar cell lesions and associated ductal hyperplasia in presence of different tumors types.

Tumor types[(**)]	**CCC(%)**		**CCH(%)**		**FEA(%)**		**Total(%)**
	DH	***ADH***	***DH***	***ADH***	***DH***	***ADH***	
invasive carcinomas	3 (11.5)	3 (11.5)	1 (50.0)	1 (50.0)	-	3 (17.6)	11 (24.4)
ductal carcinoma in situ	2 (7.7)	2 (7.7)	-	-	3 (17.6)	4 (23.5)	11 (24.4)
benign tumors	10 (38.5)	4 (15.4)	-	-	1 (5.9)	1 (5.9)	16 (35.6)
without tumor	2 (7.7)	-	-	-	1 (5.9)	4 (23.5)	7 (15.6)
Total	17 (65.4)[(*)]	9 (34.6)	1 (50.0)	1 (50.0)	5 (29.4)	12 (70.6)[(*)]	45 (100)

[(*)]Flat epithelial atypia is often associated with ductal hyperplasias with atypia (ADH) and columnar cell changes witout atypia are more frequent in hyperplasias without atypia (UDH) (19/30) (P<0.05).
[(**)]Misdorp,1999. *International Histological Classification of Tumors of Domestic Animals*.
CCC: columnar cell change; CCH: columnar cell hyperplasia without atypia; FEA: flat epithelial atypia; DH: ductal hyperplasia without atypia; ADH: atypical ductal hyperplasia;

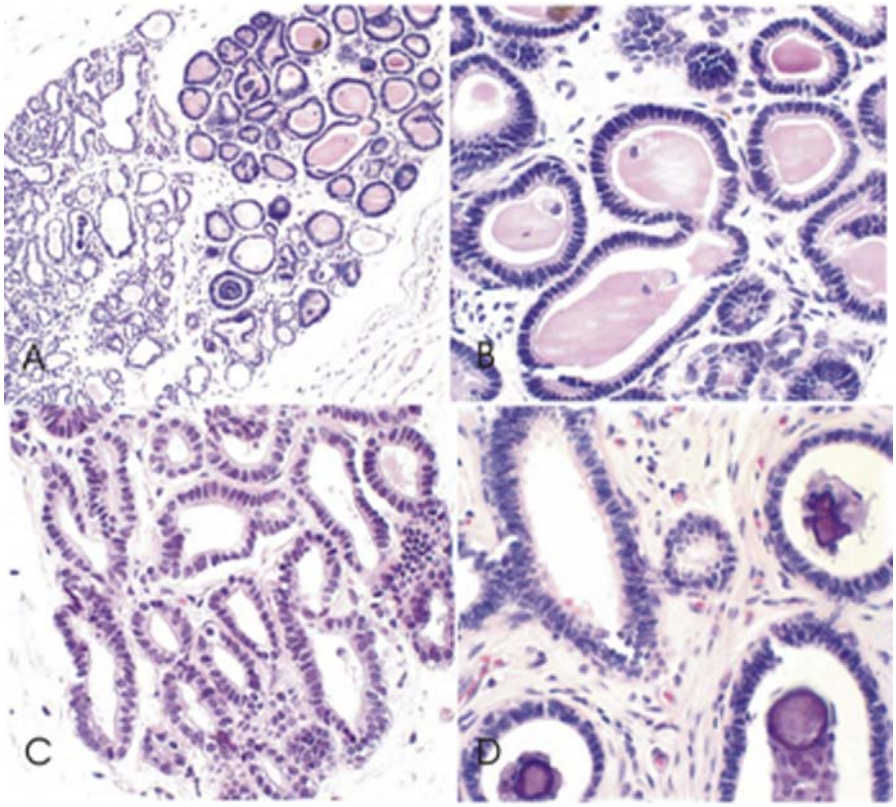

Figure 1: **Canine mammary biopsies with columnar cell change without atypia (CCC) H&E stain. 1A** Low-power view, showing dilated mammary acini in a TDLU lined with a single layer of epithelial columnar cells with underlying myoepithelial cells, many of which contain intraluminal secretions and many hyperplastic foci, 200X**; 1B, 1C e 1D** At higher magnification, the lining columnar cells with uniform ovoid to elongated nuclei and nucleoli absent. Many contain intraluminal secretions (**1B**) and show a small apical snout (**1C)** and intraluminal calcifications **(1D)**, 600X.

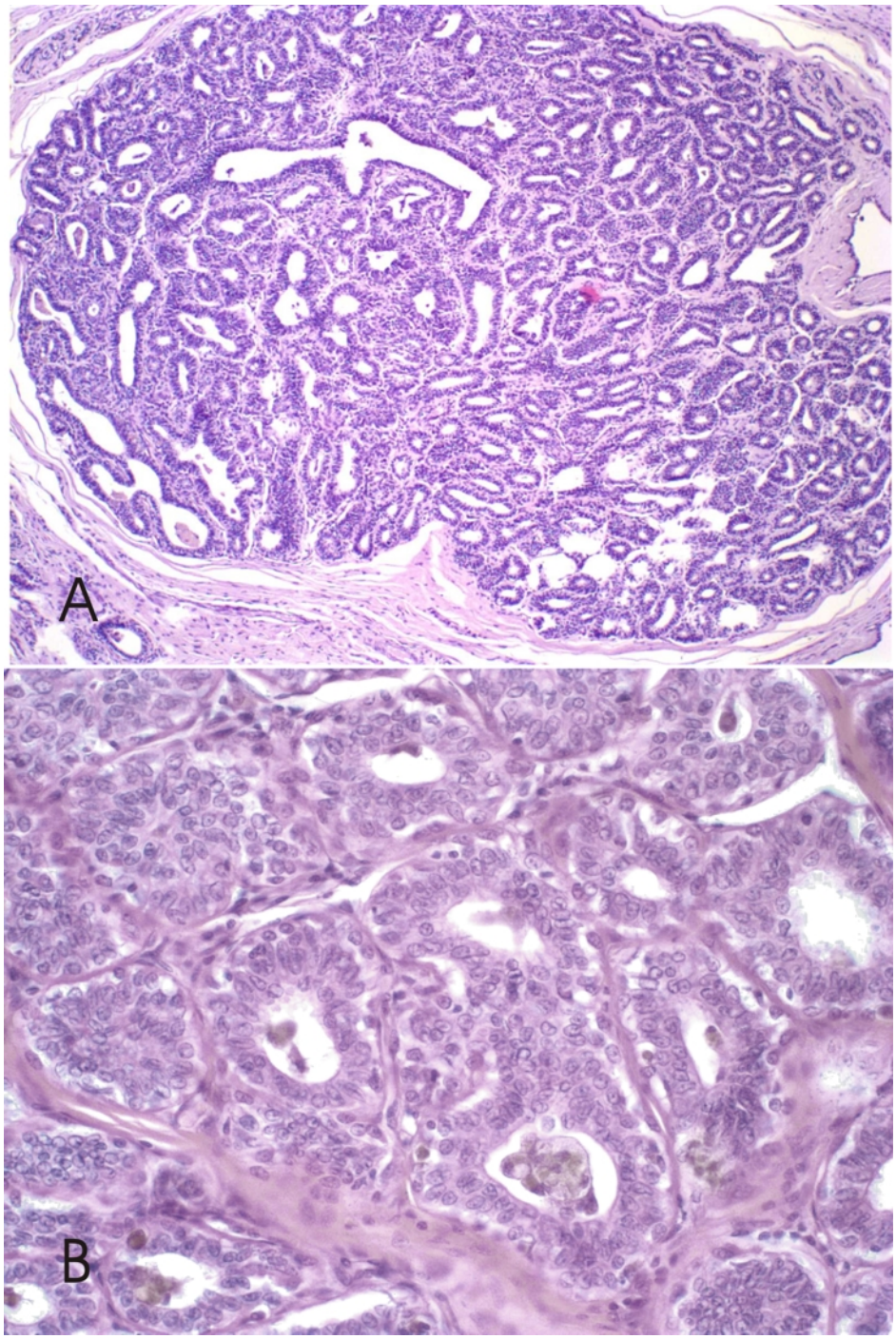

Figure 2: **Canine mammary biopsies with columnar cell hyperplasia without atypia (CCH) H&E stain. 2A** Low-power view, terminal duct lobular unit with cellular stratification, more than two cell layers, 100X. **2B** Higher power view; columnar cells with uniform ovoid to elongated nuclei, observed hobnail cells with nucleoli absent or inconspicuous, 600X.

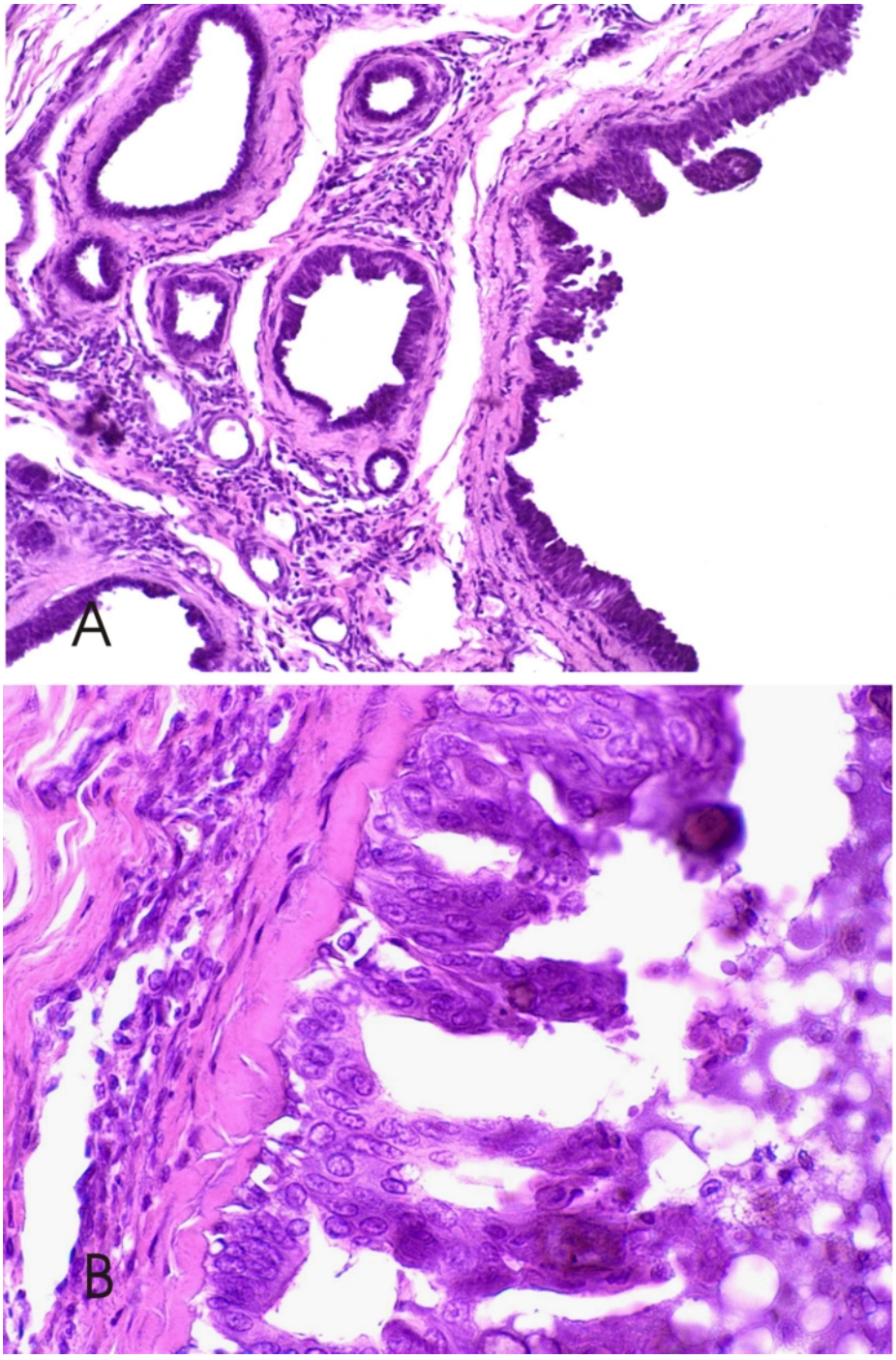

Figure 3 **Canine mammary biopsies with flat epithelial atypia (FEA) H&E stain. 3A** Low-power view; terminal duct lobular unit shows larger acini with cellular atypia, prominent apical cytoplasmic snouts, to be lined by >2 cell layers. 200X; **3B** Higher power view; the columnar epithelial cells (> 2 layers) lining the acini with intraluminal secretions show cytological atypia, characterized by apical cytoplasmic snouts and enlarged, uniform nuclei; the nuclear/cytoplasmic ratio is increased. Nucleoli are evident in some of the nuclei, 600X.

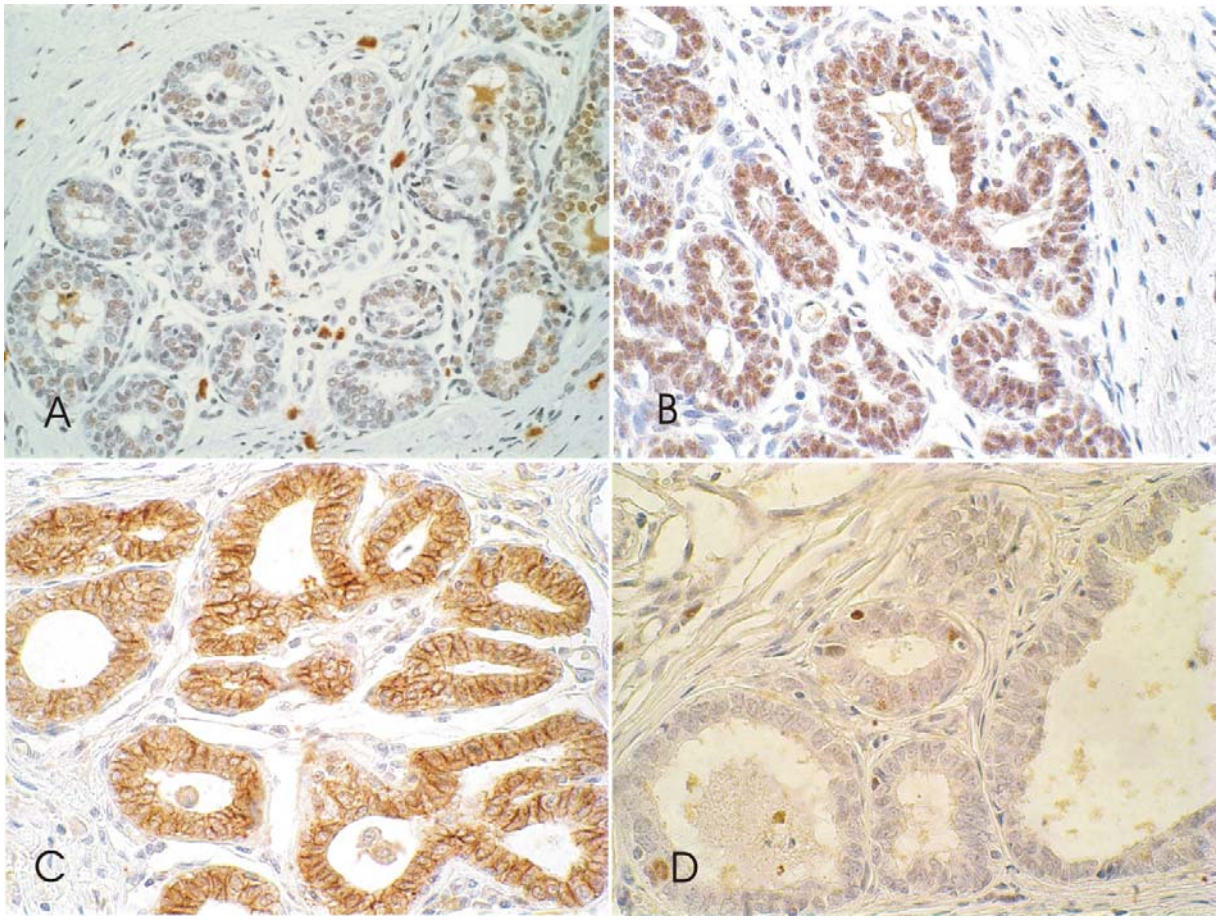

Figure 4: **Canine mammary biopsies with immunohistochemical staining of columnar cell lesion (CCL).** Columnar cell change without atypia showing intense immunostaining for estrogen receptor (**4A)** and progesterone receptor **(4B)**. Flat epithelial atypia with moderate membrane E-cadherin reactivity **(4C)** and nuclear expression for Ki-67 **(4D),** 600X.

ARTIGO 2

Evidence of molecular alterations in canine mammary ductal hyperplasia with and without atypia: morphological and immunohistochemical study

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Molecular alterations in canine mammary ductal hyperplasia with and without atypia: morphological and immunohistochemical study

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Abstract

This study describes the histologic similarity between human and canine mammary ductal hyperplasias and evaluates the morphologic and molecular relationship between ductal hyperplasias with and without atypia and canine mammary neoplasias. One hundred and fifteen cases, with previous diagnosis of intraepithelial lesions, were selected from the archives of the Laboratory of Comparative Pathology of the Biological Science Institute of the Federal University of Minas Gerais. Twenty cases of ductal hyperplasias were selected for the immunohistochemical analysis for HER2, EGFR, P53, E-cadherin, ER, PR, Ki-67 and cytokeratins 1, 5, 10 and 14 (CK34βE12). Ductal hyperplasias were identified associated with malignant neoplasias in 56 (48,8%) cases. Ductal hyperplasia without atypia was the most frequently found type of hyperplasia. however most hyperplasias with atypia were associated with mammary cancer. The ER, E-cadherin, and CK34βE12 expressions were quite low and HER2 overexpression was absent in ductal hyperplasias. Only one atypical ductal hyperplasia and associated ductal carinoma *in situ* were positive for p53. The proliferation rate (Ki-67 expression), EGFR, and PR expression appeared higher in the hyperplastic lesions analyzed than in normal mammary glands. The results showed that the immunoexpression of these markers, except for EGFR, is more evident in ductal hyperplasias with atypia when compared with hyperplasias without atypia. There was no significant difference in EGFR expression between ductal hyperplasias with or without atypia. These findings suggest that canine mammary atypical hyperplasias may play an important role in the process of malignant neoplastic transformation, show molecular alterations, similar to precursor lesions of the human breast.

Key-words: female dog, intraepithelial lesions, neoplasm, immunohistochemistry

Introduction

The canine mammary gland bears a wide range proliferative epithelial lesions similar to the human breast. Epithelial lesions can be divided into two major groups: non-neoplastic and neoplastic[35, 34, 32]. Among the canine mammary neoplasias, carcinomas in benign mixed tumors and adenocarcinomas are considered most prevalent and are noted for their negative influence on the clinical course of animals[20, 11].

Epithelial non-neoplastic lesions, such as ductal hyperplasia (DH) and lobular hyperplasia (LH) are frequently diagnosed in canine mammary gland[26]. These lesions are influenced by sex hormones and prolonged exposure to exogenous progestins, given for oestrus prevention[8, 19, 25, 45]. There is no clinical evidence that canine mammary hyperplastic lesions are precancerous. However, in humans, breast hyperplasias are recognized as risk factors for invasive cancer [31, 12, 15].

Several biological markers are expressed in different phases of mammary carcinogenesis. Few attempts have been made to correlate biologic markers in canine preneoplastic lesions with morphological criteria and associated malignant lesions[10, 13, 43].

Although epidemiological studies in dogs have limitations in demonstrating the premalignant potential of mammary hyperplasia, recent studies suggest that some hyperplasias may have molecular features indicative of neoplastic transformation[37, 2]. In spite of these aspects, the precise anatomic definition of these lesions has been proposed only rarely and their biologic or prognostic significance has never been rigorously studied.

The present work aimed to stratify the pathologic spectrum of canine mammary hyperplasias, compared with the classification used in human pathology. We evaluated molecular markers (ER, PR, p53, Ki-67, EGFR, HER-2, high-molecular-weight cytokeratins (34βE12) and E-

cadherin) in epithelial hyperplasias of mammary specimens with or without an association to carcinomas.

Methods

We evaluated histologic sections from 115 epithelial hyperplasias from canine breast lesions selected from the archives of the Laboratory of Comparative Pathology of the Biological Science Institute of Federal University of Minas Gerais. The mammary gland samples were obtained after clinical diagnosis of mammary tumor and surgical removal of the lesion.

Hematoxylin and eosin–stained sections were reviewed to search for epithelial hyperplasias and associated epithelial tumors. Slides of a total of 115 biopsy specimens of lesions were examined and classified by three pathologists. One human pathologist (HG) and two veterinary pathologists (EF and GDC) individually reviewed and classified the hyperplastic lesions using the human[41] and veterinary classification[26]. The canine mammary neoplasias were classified according to veterinary nomenclature[26].

According to WHO veterinary classification, epithelial hyperplastic changes were divided into two categories: intralobular ductal hyperplasia (LH) and interlobular ductal hyperplasia (DH), including subclassification based on the presence of cytological atypia (LHA and DHA) [26]. When both, LH and DH lesions were found in the same specimen, we grouped these lesions under the category of “mixed” hyperplasia without atypia, or mixed hyperplasia of usual type (MHU) and mixed hyperplasia with atypia (MHA) for purposes of analysis.

The human criteria used to classify ductal hyperplasia were those from WHO human classification[41]. The lesions were classificated as ductal hyperplasia without atypia (hyperplasia usual type - HUT) or atypical ductal hyperplasia (ADH).

Considering the small size of the lesions, the immunohistochemical analyses were performed only on cases with enough material. Paraffin blocks were selected from twenty cases containg hyperplastic lesions classified by WHO human criteria (10 ADH and, 10 HUT). ADH were associated with three carcinomas arising in benign mixed tumors, four ductal carcinomas in situ (DCIS), and three benign mixed tumors. HUT were associated with one carcinoma arising in benign mixed tumors, three DCIS, four benign mixed tumors and two duct papilloma.

Consecutive 5 μm thick sections were obtained and mounted on silanated slides for the immunohistochemical study. Sections were stained for rabbit polyclonal antibodies: HER2 (c-erbB-2; Dako; dilution: 1:40), EGFR (clone 31G7, Zymed, diluition: 1:100); P53 (clone CM1; Covance; dilution: 1:80); and mouse monoclonal antibodies: E-cadherin (clone 4A2C7; Zymed; dilution: 1:100), ER-LH2 (clone CC4-5; Novocastra; dilution: 1:25), PgR (clone hPRa2; Neomarkers; dilution: 1:20), Ki-67 (clone Mib-1; Dako; dilution: 1:25), cytokeratins 1, 5, 10 and 14 (clone 34βE-12; Dako; dilution: 1:40). Heat-induced epitope retrieval (20 min) using Dako antigen retrieval solution, pH 6.0 (Dako), was previously performed in a water bath. The slides were then cooled to room temperature for 20 min in the antigen retrieval buffer. The sections were incubated for 15 min at room temperature in 3% (vol/vol) H_2O_2, in primary antibodies for 16 h, in reagent contained anti-mouse and anti-rabbit secondary antibodies (Biotinylated Goat Anti-polyvalent, Laboratory Vision) for 15 min and streptavidin peroxidase (UltraVision Large Volume Detection System, HRP, Laboratory Vision) for 15 min. Between incubations, the slides were washed for 2 x 5 min in phosphate-buffered saline containing 1% (vol/vol) Tween 20. Immunoreactivity was visualized by incubating the slides for 10 min with diaminobenzidine (DAB Substrate System; Laboratory Vision). The slides were then counterstained with Harris hematoxylin. Positive and negative control slides were included in each batch. As a positive control we used human breast cancer tissue known to

express the antibodies. Negative controls were assessed using normal serum (Ultra V Block, Laboratory Vision) as the primary antibody.

Staining for cytokeratin 34βE12 and E-cadherin was evaluated semi-quantitatively and scored into five categories: negative (-), <5% of cells stained; positive (+), 5% to 25% of cells stained; positive (++), 25% to 50% of cells stained; positive (+++), 50% to 75% of cells stained; diffusely positive (++++), >75% of epithelial cells stained. For ER and PR, first, a proportion score was assigned, which represented the estimated proportion of positive-staining tumor cells (0, none; 1, < 1⁄100; 2, 1⁄100 to 1⁄10; 3, 1⁄10 to 1⁄3; 4, 1⁄3 to 2⁄3; and 5, > 2⁄3). Next, an intensity score was assigned, which represented the average intensity of positive tumor cells (0, none; 1, weak, 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8[17]. Cases were classified as ER and PR positive when the total score was equal to or greater than 3. The p53 staining was considered positive when more than 10% of cells exhibited positive nuclear staining, independent of staining intensity. The proliferative index was calculated by counting the nuclei positive for Ki-67 staining in a total of 500 epithelial cells from each lesion. HER2 and EGFR expressions were defined as epithelial cell membrane staining and scored according to the American Society of Clinical Oncology and College of American Pathologists[46].

Immunohistochemical data was analyzed with nonparametric methods on semiquantitative IHC scores using GraphPad InStat®. The Kruskal–Wallis test was used to compare groups (HUT, ADH, mammary tumors, and normal mammary glands) for each antibody. Associations between categorical variables (ER, EGFR) in HUT and ADH, were assessed by Fisher's exact test. Statistical results were considered significant when $P<0.05$. Morphologic lesions were expressed with descriptive statistics. All procedures were performed under the guidelines and with the approval of the Ethics Committee for Animal Experimentation (CETEA/UFMG), protocol 192/2006.

Results

The histologic review showed that ductal hyperplasias were associated with other mammary tumors in the majority of the cases (98/115; 85.21%). It was possible to determine the age of animals and the affected mammary gland in 68 and 53 cases respectively. The age of the animals at the time of surgery ranged from 3 to 16 years (mean 9.5 years ± 2.5).The lesions were more frequent in the abdominal mammary gland (33 cases; 49.3%) and inguinal gland (20 cases; 29.8%) than in thoracic glands (14 cases; 20.9%).

Ductal hyperplasias were identified when ducts showed increased cellularity and appeared to be composed mostly of ductal epithelium and, often, myoepithelium, both of which closely resembled normal epithelium throughout the gland, according WHO human and veterinary classification[26,41]. Hyperplastic changes were more commonly seen within small intralobular ductules and interlobular ducts and sometimes the hyperplasias were also seen in larger ducts.

Usual ductal hyperplasia was diagnosed when increased ductal epithelial cellularity was seen, but without cytological or architectural atypia. Affected ducts were partially filled by irregularly shaped fenestrations lined by epithelial cells without polarity. Cords of epithelial cells that sometimes bridged the ductal lumina were found, and epithelial cells often seemed to overlap because cytoplasmic boundaries were indistinct (Figure 1) [26,41].

The atypical ductal hyperplasia was characterized by solid or papillary proliferations of small, dark, cuboidal to low columnar cells, often forming parallel rows with a palisandig or basaloid cell apearence (Figure 2) [26,41]. Using Schnitt's *et al.* criteria, ADH was distinguished from low- or intermediate grade DCIS by the rudimentary and irregular shape of fenestrations and cytologic grade of atypia. In lesions with low-grade cellular atypia, the size of the proliferation

was also considered. Whereas high-grade cellular atypia always warranted the classification of high-grade DCIS, if features of low- or intermediate-grade DCIS were observed, but did not involve more than one duct or space the intraepithelial lesion was classified as ADH[36].

A total of 98 mammary epithelial neoplasms were diagnosed in females dogs, 42 benign and 56 malignant. The most prevalent neoplasms were DCIS (20 cases), carcinomas arising in benign mixed tumors (18 cases), benign mixed tumors (19 cases) and ductal papillomas (13 cases), in that order. Independent of the use of human or veterinary classification, the mammary carcinomas were more often observed with the atypical hiperplasias. However, when to using the WHO veterinary classification, among both the benign and malignant neoplasms the percentages of the specific types were similar among the intralobular ductal hyperplasia, interlobular ductal hyperplasia or mixed hyperplasia. Table 1 summarize the occurrence of specific ductal hyperplasias and epithelial mammary neoplasms included in this study, according veterinary classifications.

According human classification, of the 51 dogs with ADH, 30 (58,8%) had malignant mammary tumors and only 12 (23,5%) had benign neoplasms. Table 2 summarize the occurrence of specific ductal hyperplasias, the mammary carcinomas were more often associated with ADH ($p<0,05$).

Immunohistochemical stainings showed positive cytoplasmic localization of cytokeratin 34βE12 in all hyperplastic cells adjacent to or distant from the mammary tumors, but less intense than in normal mammary epithelium ($p<0,05$) (Figure 3 and 4). E-cadherin expression showed the same pattern of immunostaining in mammary hyperplastic lesions and normal ducts. In ductal hyperplasias and mammary tumours, E-cadherin staining was observed in both benign and malignant epithelial cells. However, luminal epithelial cells usually showed a reduced level of expression, 4 cases were negative (2 HUT and, 2 ADH)

(p<0,01) (Figure 5 and 6). No significant difference was observed between E-cadherin or cytokeratin 34βE-12 expression in cells of HUT, ADH and DCIS.

The expression pattern of cell cycle related proteins (ER, PR, p53, EGFR, HER2, and Ki-67) was variable among ductal hyperplasias and the adjacent tumors. The ER immunostaining was localized in the nuclei and showed some variability in intensity even in individual lesions of the same case, with decreased expression in HUT and ADH compared to normal mammary gland (p<0,01) (Figure 7 and 8). Only three cases of the HUT and one of ADH were positive for ER. The majority of the epithelial cells of HUT and ADH in all specimens showed positive staining for PR, and high expression of Ki-67 and EGFR. Statistical analysis of the scoring of EGFR staining intensity revealed significant differences among HUT, ADH, benign tumors, and DCIS (P = 0.01). However EGFR overexpression was detected in only two cases of the HUT (Figure 9 and 10). The immunohistochemical results are summarised in Tables 3 and 4.

Only one atypical ductal hyperplasia and associated DCIS was positive for p53. The other three tumors were positive for p53, (one DICS, one benign tumor and one invasive carcinoma), but no evidence of the p53 expression was observed in adjacent ductal hyperplasias. Two invasive carcinomas showed HER2 overexpression, however all proliferative cells of ductal hyperplasias with or without atypia adjacent to tumors were negative for HER2.

Discussion

Apparently, hyperplastic lesions in dogs follow a morphological pattern similar to that seen in human beings. However, the current classification criteria for the epithelial hyperplasias in dogs do not correlate with possible prognostic features, as they are based only on the location and presence or absence of atypia[26].

Our results regarding presence of neoplastic and pre-neoplastic lesions, the sites they usually affect and the mean age when they occur, are similar to previous published studies[3, 32]. About 56% of non-neoplastic mammary nodules are represented by hyperplastic lesions with diffuse forms, which occur in similar proportion in inguinal, abdominal, and thoracic mammary glands[5].

In accordance with our results, ductal hyperplasias are mainly associated with mixed tumors. ADH is more commonly associated with invasive carcinomas. Our findings suggest that ADH is precursor lesions of invasive carcinomas of canine mammary gland similar to human breast[30, 31, 24, 33]. Morphological and epidemiological studies of human breast previously showed an increased risk of progression of atypical ductal hyperplasias toward ductal carcinoma in situ and invasive carcinomas[29, 42].

In our study, the high frequency of hyperplasias associated with neoplastic lesions may be because of criteria used for case selection. Our samples were obtained from animals submitted to mammary biopsy indicated by nodule or tumor. However, atypical hyperplasias were more frequently associated with mammary carcinomas (in 30 out of 51 cases) compared to association with benign tumors.

Data obtained from human and canine mammary studies support the hypothesis that hyperplastic lesions with and without atypia and the carcinogenesis process originate from cumulative genetic changes in the cells[25].

The expression of different prognostic molecular markers has already been shown in neoplastic and non-neoplastic lesions of canine mammary glands. There is a direct relationship between the expression of oncogenes and proteins involved in the process of neoplastic transformation and the malignant nature of the lesion[10, 23, 37].

ADH and DCIS of human breast show low expression of high-molecular-weight cytokeratins CK5, CK14, and CK903[28, 21]. In our study, we obtained similar results with the 34βE12 staining in ADH and DCIS of canine similar lesions. Our findings suggest that during the malignant progression the epithelial cells lose the 34βE12 expression. In addition to its possible role in cancer progression, the high molecular 34βE12 staining may also be a useful tool to differentiate HUT and ADH/DCIS, similar to its use in human breast biopsies.

Bratthauer et al. suggested that the combination of 34βE12 and E-cadherin antibodies could be useful in distinguishing lobular and ductal lesions that have overlapping morphological features[4]. In addition to differentiating ductal lesions, a significant reduction of E-cadherin expression in atypical intraepithelial lesions may give relevant information about the unfavorable prognostic feature of certain mammary changes (ADH and DCIS)[44]. This decreased E-cadherin expression has a direct relationship with the increase of cell proliferation (Ki-67 index) and reduction of ER expression, as seen in our results. It might be explained by transmembrane signaling mechanisms of growth factor receptors, hormone receptors, and molecules associated to epithelial-mesenchymal transition[1, 16].

The ER-estrogen complex is known for inducing genetic and epigenetic changes and for influencing different genes involved in the regulation of cell differentiation and proliferation[39]. From these mechanisms, an epithelial-mesenchymal transition (EMT) is suggested, as well as the malignization of mammary epithelial lesions is thought to be associated with a regulation of E-cadherin expression mediated by estrogen receptor signaling[47]. There are few studies elucidating how this relationship occurs. However, there is a direct relationship between loss of E-cadherin expression and hormone receptors and increased expression of Ki-67 in breast cancer[18]. There are no data to suggest direct functional interaction between Ki67 and ER or E-cadherin proteins, so the strong association of theses markers with poor prognosis is uncertain.

Previous results lead us to hypothesize that p53 may regulate ER expression. The ER negativity in mammary cancers seems to be related to poor prognosis associated with a P53 positivity[40]. In our series, the tumors cases with p53 expression were ER negative, and one case of ADH with DCIS associated were p53+. The occurrence of p53 mutants by chance during neoplastic transformation cannot be excluded, however our results should be interpreted with caution. The interpretation of these results is of course speculative, more direct evidence of our hypothesis could be obtained from the prospective study of dogs with ADH or DICS p53 positivity.

The ER activation process stimulates DNA synthesis, cell division and the production of biologically active proteins, including transforming growth factor (TGF) and epidermal growth factor (EGF) which influence cell growth and differentiation[7, 38]. Atypical intraepithelial lesions in canine mammary glands apparently have a reduced ER expression, and absence of EGFR and HER2 overexpression. These may be related to the activation of cell clones with loss of differentiation characteristics associated with the reduction of the existing regulation between ER co-expression and growth factors. Our results suggest that, in ER-negative mammary lesions, cell proliferation is probably controlled by other paracrine growth factors (TGF-α and TGF- β) released by ER-positive cells and should not be controlled by the autocrine ER activation of cyclins associated with the EGFR overexpression[6, 9].

The absence of ER expression in ductal hyperplasias and DCIS, which was observed in 75% of our cases, suggests that these lesions may be associated with the tumor development with a negative expression of HER2 and ER, similar to what has been described in previous studies. On the basis of our data and in accordance with other authors, canine mammary intraepithelial lesions may become an interesting model to further study HER2 and ER negative mammary tumors[2, 27].

In conclusion, epithelial hyperplasias of canine mammary glands are seen in the same ductal-lobular organization and have similar morphological and molecular characteristics to similar human lesions. We observed a direct relationship between the presence of in situ and invasive mammary carcinomas and the occurrence of atypical ductal hyperplasias. Gradual loss of expression of proteins related to proliferation control (ER, EGFR) and E-cadherin in intraepithelial lesions, suggests that mammary hyperplasias of canine may play an important role in the process of malignant neoplastic transformation. Prospective studies evaluating expression of molecular markers in epithelial proliferations and clinical evaluation may contribute to a better understanding of these changes in canine mammary cancer development.

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Table 1: Distribution of types of ductal mammary hyperplasias and associated tumors according to veterinary pathology classification[26].

Tumor types (*)	*DHU(%)*	*DHA (%)*	*LHU(%)*	*LHA(%)*	*MHU(%)*	*MHA(%)*	*Total(%)*
invasive carcinoma	**5(25.0)**	**5(38.4)**	**3(12.5)**	**3(27.3)**	**3(15.0)**	**7(25.9)**	**26(22.6)**
carcinoma in benign tumor	*5(25.0)*	*4(30.7)*	*3(12.5)*	*2(18.2)*	-	*5(18.5)*	
solid carcinoma	-	-	-	*1(9.1)*	*2(10.0)*	-	
tubulopapillary carcinoma	-	*1(7.7)*	-	-	*1(5.0)*	*2(7.4)*	
***in situ* carcinomas**	**6(30.0)**	**4(30.8)**	**6(25.0)**	**3(27.3)**	**3(15.0)**	**8(29.7)**	**30(26.2)**
"in situ" carcinoma in benign tumor	*1(5.0)*	*1(7.7)*	*4(16.7)*	*1(9.1)*	-	*3(11.2)*	
ductal carcinoma "in situ"	*5(25.0)*	*3(23.1)*	*2(8.3)*	*2(18.2)*	*3(15.0)*	*5(18.5)*	
benign tumors	**7(35.0)**	**3(23.1)**	**13(54.2)**	**2(18.2)**	**9(45.0)**	**7(25.9)**	**41(35.6)**
simple adenoma	*1(5.0)*	1(*7.7*)	*3(12.5)*	-	*3(15.0)*	*1(3.7)*	
duct papilloma	*2(10.0)*	1(*7.7*)	*3(12.5)*	*1(9.1)*	*4(20.0)*	*2(7.4)*	
benign mixed tumor	*4(20.0)*	1(*7.7*)	*7(29.2*)	*1(9..1*)	*2(10.0*)	*4(14.8*)	
without tumor	**2(10.0)**	**1(7.7)**	**2(8.3)**	**3(27.3)**	**5(25.0)**	**5(18.5)**	**18(15.6)**
Total	**20(100)**	**13(100)**	**24(100)**	**11(100)**	**20(100)**	**27(100)**	**115(100)**

(*)Misdorp *et al.*, 1999. *Histological Classification of Mammary tumors of the dog and the cat.*
DHU: ductal hyperplasia without atypia; DHA: ductal hyperplasia with atypia; LHU: lobular hyperplasia without atypia; LHA: lobular hyperplasia with atypia; MHU: mixed hyperplasia without atypia; MHA: mixed hyperplasia with atypia

Table 2: Distribution of types of ductal mammary hyperplasias and associated tumors according to human classification. The mammary carcinomas were more often associated with the ADH ($p<0.05$).

Tumors types (*)	*HUT(%)*	*ADH (%)*	*Total(%)*
invasive carcinomas	**11(17.2)**	**15(29.4)**	**26(22.6)**
carcinoma in benign tumor	*8(12.5)*	*11 (21.6)*	
solid carcinoma	*2(3.1)*	-	
tubulopapillary carcinoma	*1(1.6)*	*4(7.8)*	
***in situ* carcinomas**	**15(23.4)**	**15(29.4)**	**30(26.1)**
"in situ" carcinoma in benign tumor	*5(7.8)*	*5(9.8)*	
ductal carcinoma "in situ"	*10(15.6)*	*10(19.6)*	
benign tumors	**29(45.3)**	**12(23.5)**	**41(35.7)**
simple adenoma	*7(10.9)*	*2(3.9)*	
duct papilloma	*9(14.1)*	*4(7.8)*	
benign mixed tumor	*13(20.3)*	*6(11.8)*	
without tumor	**9(14.1)**	**9(17.7)**	**18(15.6)**
Total	**64(100)**	**51(100)**	**115 (100)**

(*)Misdorp *et al.* ,1999. *Histological Classification of Mammary tumors of the dog and the cat.*
HUT: hyperplasia of usual type; ADH: atypica ductal hyperplasia.

Table 3: Average expression of KI-67, EGFR, ER, PR, E-cadherin, and 34βE12 in normal mammary epithelium, HUT, and tumors from the same mammary specimens.

	Normal mammary epithelium	HUT	Mammary tumors	*P*
KI-67	25.09 ± 27.97 (a)	32 ± 23.68 (a)	27.44 ± 22.59 (a)	0.87
EGRF	0.3 ± 0.48 (a)	1 ± 1.24 (a)	2.3 ± 0.7 (b)	<0.01
ER	3.5 ± 0.52 (b)	1.08 ± 1.16 (a)	1 ± 0.94 (a)	<0.001
PR	5.77 ± 0.66 (b)	4.75 ± 0.46 (a)	4 ± 1.32 (a)	<0.001
E-cad	4 ± 0 (b)	1.9 ± 1.16 (a)	2.2 ± 1.06 (a)	<0.01
34βE12	3 ± 0 (a)	2.72 ± 0.46 (a)	1.9 ± 1.22 (b)	<0.01

HUT: hyperplasia usual type. Equal letters on the same line have not differed significantly ($p<0.05$)

Table 4: Average expression of KI-67, EGFR, ER, PR, E-cadherin, and 34βE12 in normal mammary epithelium, ADH, and tumors from the same mammary specimens.

	Normal mammary epithelium	ADH	Mammary tumors	*P*
KI67	22.16 ± 0.5 (a)	37.83 ± 17.09 (ab)	62 ± 35.46 (b)	0.04
EGRF	0.5 ± 0.55 (a)	0.67 ± 0.82 (b)	1.83 ± 0.98 (b)	<0.01
ER	3.72 ± 0.46 (b)	0.90 ± 0.7 (a)	1 ± 1.54 (a)	<0.01
PR	6 ± 0 (b)	4.88 ± 0.6 (a)	4.57 ± 0.78 (a)	<0.01
E-cad	4 ± 0 (b)	1.87 ± 1.24 (a)	2.83± 1.6 (ab)	<0.01
34βE12	3 ± 0 (a)	2.5 ± 0.7 (a)	2.5 ± 0.52 (a)	0.05

ADH: atypical ductal hyperplasia. Equal letters on the same line have not differed significantly ($p<0.05$)

Fig. 1. Mammary gland; *Canis familiaris*. Case 18. Ductal hyperplasia without atypia showing pleomorphic cells with micropapillary projections and irregular intraductal bridge formations (arrow). 400x. Hematoxylin and Eosin.

Fig. 2. Mammary gland; *Canis familiaris*. Case 18. Ductal hyperplasia with atypia presenting cells with a monomorphic pattern with regular intraductal bridge formations (arrow). 400x. Hematoxylin and Eosin.

Fig. 3. Mammary gland; *Canis familiaris*. Case 67. Usual ductal hyperplasia showing intense immunostaining for cytokeratin 34*β*E12. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

Fig. 4. Mammary gland; *Canis familiaris*. Case 18. Atypical ductal hyperplasia with moderate cytokeratin 34*β*E12 expression. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

Fig. 5. Mammary gland; *Canis familiaris*. Case 67. Usual ductal hyperplasia showing strong membrane and cytoplasmatic E-cadherin reactivity. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

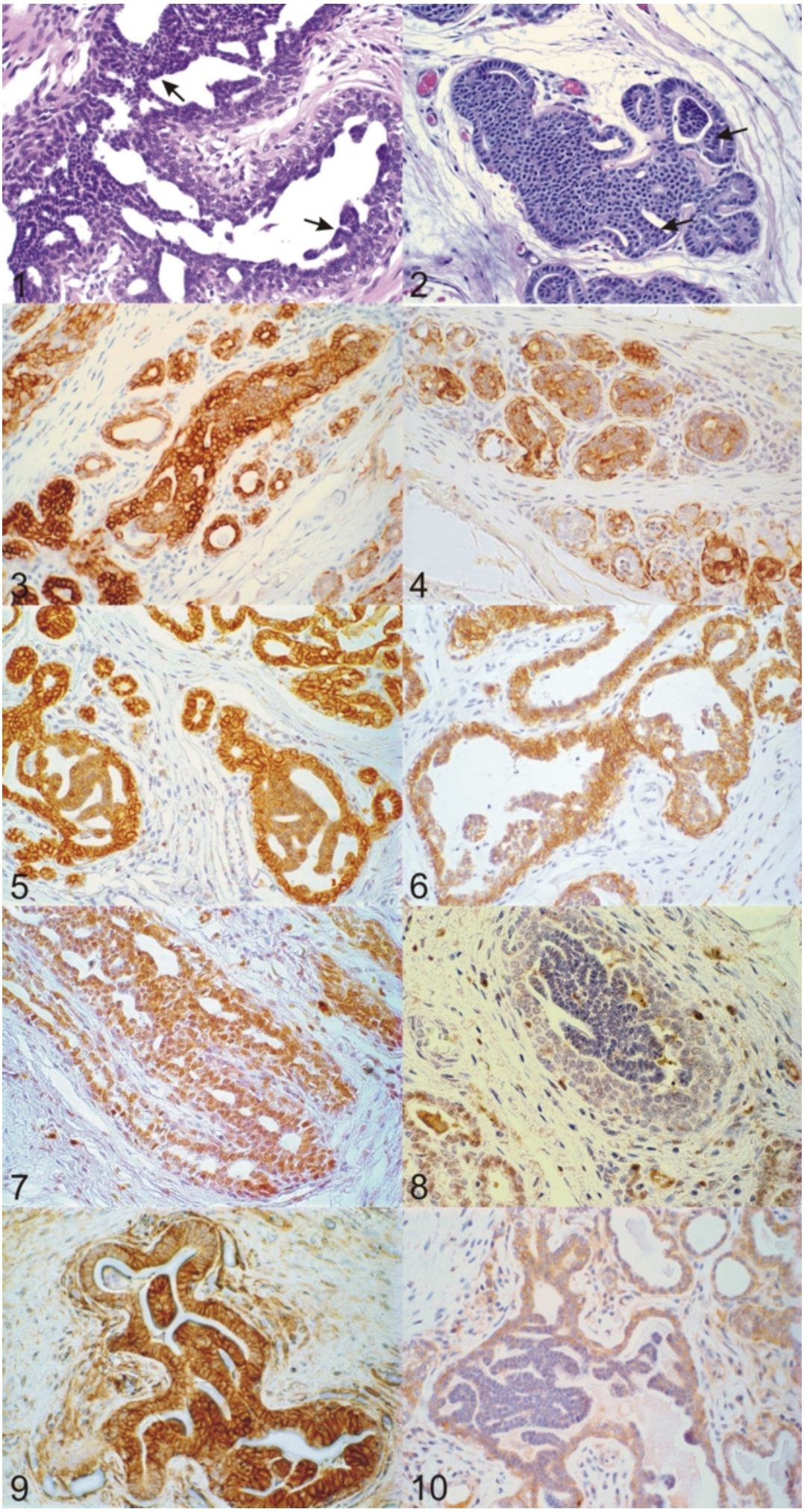
Fig. 6. Mammary gland; *Canis familiaris*. Case 15. Atypical ductal hyperplasia with lower concentration of membrane and cytoplasmatic E-cadherin immunoreactivity. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

Fig. 7. Mammary gland; *Canis familiaris*. Case 24. Usual ductal hyperplasia showing moderate immunostaining for estrogen receptor in the nuclei of epithelial cells 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

Fig. 8. Mammary gland; *Canis familiaris*. Case 55. Atypical ductal hyperplasia negative for estrogen receptor. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

Fig. 9. Mammary gland; *Canis familiaris*. Case 67. Usual ductal hyperplasia showing intense immunostaining for epidermal growth factor receptor. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.

Fig. 10. Mammary gland; *Canis familiaris*. Case 15. Atypical ductal hyperplasia showing lack expression for epidermal growth factor receptor. Spindle cells in their surroundings are positive. 400X. Advance HRP Visualization Method, Harris's hematoxylin counterstain.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ARTIGO 3

HER-2 gene and protein expression in atypical ductal hyperplasia associated with canine carcinomas in benign mixed tumors

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HER-2 gene and protein expression in atypical ductal hyperplasia associated with canine carcinomas in benign mixed tumors

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Abstract

From among members of the epidermal growth factor receptors family, HER2 protein is related to cell proliferation, motility and survival, with its gene amplification being the main mechanism responsible for its overexpression in mammary tumors. The present study has the purpose of reporting the behavior of HER2 gene and the expression of its protein in atypical ductal hyperplasias associated to HER2-positive canine mammary carcinomas. Three histological samples of canine mammary mixed tumors with an HER2 overexpression were selected with the use of chromogenic in situ hybridization (CISH) technique, the behavior of HER2 gene was evaluated in these carcinomas and in the associated HDA. Apparently, a weak or no expression was observed for HER2 protein in atypical hyperplasias as well as the absence of amplification of its coding gene in carcinomas in canine mammary mixed tumors and in lesions associated to these tumors. The behavior of HER2 protein and its gene in canine mammary carcinomas is similar to some subtypes of human mammary tumors, and supports the proposal of using a canine model in studies related to new therapeutic perspectives for HER2-positive tumors with no gene amplification.

Key words: dog, intraepithelial lesions, neoplasm, immunohistochemistry

Different types of non-invasive epithelial mammary lesions are associated to a risk of developing breast cancer in the human and canine species. The process of malignant neoplasm progression is observed in canine benign mixed mammary tumors, where the development of carcinomatous foci (carcinomas in situ) may result in formation of invasive carcinomas associated to these tumors. 3

The appearance of canine mammary cancer is thought to occur from molecular transformations derived from genomic instabilities experienced by usual hyperplasias (HDU), atypical hyperplasias (ADH) and ductal carcinomas in situ (DCIS). 8, 10

Cytogenetic evaluations of canine mammary tumors have shown that the chromosomal region 1q13.1, where HER2 gene is located in this species, is very affected by chromosomal aberrations.11 Studies of chromogenic in situ hybridization (CISH), in different mammary dysplasias and neoplasms, have not shown any relation between the overexpression of HER2 and the gene amplification of HER2 in canine mammary cancer.7 Nevertheless, the purpose of the change in molecular behavior regarding the process of neoplastic progression still remains unclear.

Therefore, in the present work we aim to investigate the relation between the expression of HER2 protein and the HER2 gene status by means of the chromogenic in situ hybridization (CISH) technique in three canine mammary carcinomas and in HDA associated to these lesions.

Three cases of canine mammary carcinomas were retrieved from the files of the Laboratory of Comparative Pathology, Department of General Pathology, Institute of Biological Science, Federal University of Minas Gerais, Brazil. The selection was based on histopathological diagnosis according to the World Health Organization criteria.9 All procedures were performed following the guidelines and with the approval of the Ethics Committee in Animal Experimentation.

All tissue samples were previously fixed in 10% neutral formalin and embedded in paraffin. Paraffin blocks were selected and consecutive 3 μm thick sections were obtained and mounted on silanized slides for an immunohistochemical study. Primary antibody monoclonal rabbit anti-human HER2 Oncoprotein (c-erbB-2; 1:100a) was incubated overnight (16-18hs) at 8ºC.

After incubation, immunodetection was performed with the technology to prepare polymeric HRP-linker antibodyb, with diaminobenzidine chromogen as substratea. As a positive control slide we used human breast cancer tissue known to express the antibodies. Negative controls were evaluated using normal serum as the primary antibody. HER2 expression was defined as epithelial cell membrane staining and scored according to the American Society of Clinical Oncology.16

CISH was performed using the double-stranded DNA HER2 probec according to the manufacturer's instructions and it was evaluated with the kit interpretation guidelinesc. At least 30 non-equivocal and non-overlapping neoplastic cells were counted per case. Non-amplified cases were defined as those with one to five signals per nucleus in >50% of tumor cells; amplification was defined as i) more than 5 gene copies per nucleus in >50% of tumor cells, ii) when small or large gene copy clusters were found in >50% of tumor cells.

The primary purpose of this part of the study was defined as mammary gland containing synchronous ADH in analyses of canine mammary carcinomas. For the histological classification, all three cases of invasive carcinomas were classified as carcinomas in benign mixed tumors. The malignant component is characterized by infiltrative growth, cell pleomorphism and abnormal mitoses. ADH projections were usually characterized by solid to papillary proliferations of small, dark, cuboidal to low columnar cells, often forming parallel rows with a palisading or basaloid cell appearance.

Immunohistochemical overexpression of HER2 protein (3+) was clearly in all three canine mammary carcinomas (Fig. 1A). Normal and ADH occurred in the same mammary gland all proved to be negative for HER2 (2+, 1+, 0) protein expression (Fig. 1B). Cytoplasmic staining for HER2 was found in the normal mammary epithelial cells, but not in ADH.

Criteria for sucessful CISH analysis included identifying at least one copy of the HER2 gene per nucleus in most cancer cells. CISH was successful in all three cases, however, all invasive carcinomas and associated ADH had an overexpression of the protein but had no gene amplification, showing typically one to two dots per nucleus (Figs. 1B and 1C).

In this study, HER2 positive cases was restricted to three carcinomas in mixed tumors, however, HER2 protein detection by immunohistochemical methods in formalin-fixed and

paraffin-embedded specimens has been reported in canine mammary cells. The HER2 overexpression which correlates with a more rapid progression and a worse prognosis in canine mammary cancer and is similar to that in human breast carcinoma, suggesting a possible role in carcinogenesis and working as a prognostic indicator.4,6

HER2 overexpression, in humans, is generally seen in high grade breast cancers and is associated with aggressive tumor growth characteristics including enhanced tumorigenicity. However, HER2 gene amplification occurs in approximately 20% human breast cancer patients.16 Thus, a model of mammary carcinogenesis with pathological similarities to those in humans is needed and different authors have reported the use of female dogs for prognostic and predictive evaluation of the cancer.14,15

In the invasive canine mammary cancer studied, HER2 gene amplification was not evident. Previous studies have demonstrated that human metaplastic breast carcinomas, a histological subtype with histological similarities to canine mixed tumors, with a lack of HER2 overexpression, but HER2 gene amplification was not observed.13 This fact can also be observed in other human tumors, approximately 2% of breast cancer patients express discordant HER2 protein expression independently of the HER2 gene amplification.12 HER2 transcription in the absence of gene amplification may be related to the mechanism of cell function through the enhanced production of mRNA by phosphorylation of tyrosine kinase acting on growth factors and regulators of cell growth and proliferation.5

In contrast to canine mammary cancer, the analyses of ADH presented here have not revealed the presence of HER2 protein alterations. Thus, similarly, the absence of HER2 amplification was observed in these cases. The practical importance of intraepithelial canine mammary studies is related to their potential for malignant transformation and the absence of alterations indicates that this gene may not be involved in the initial stages of proliferation. Previous studies have suggested that the overexpression of HER2 occurs prior to the development of metastatic disease in canine mammary tumors and plays a role in the development of malignancy.1,2

The use of the canine species may be the most appropriate model for progression to invasive breast cancer studies, due to the oncogenic similarities of human mammary lesions. However,

further research will be necessary to verify the usefulness of these markers as tools for the evaluation of the malignant potential of benign mammary tissue.

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Sources and manufacturers

a. DakoCytomation - Dako North America Inc., Carpinteria, CA.

b. DAKO Advance HRP Visualization Method - Dako, Glostrup, Denmark

c. Zymed's SPoT-Light® HER2 CISHTM Kit - Zymed, South San Francisco, USA

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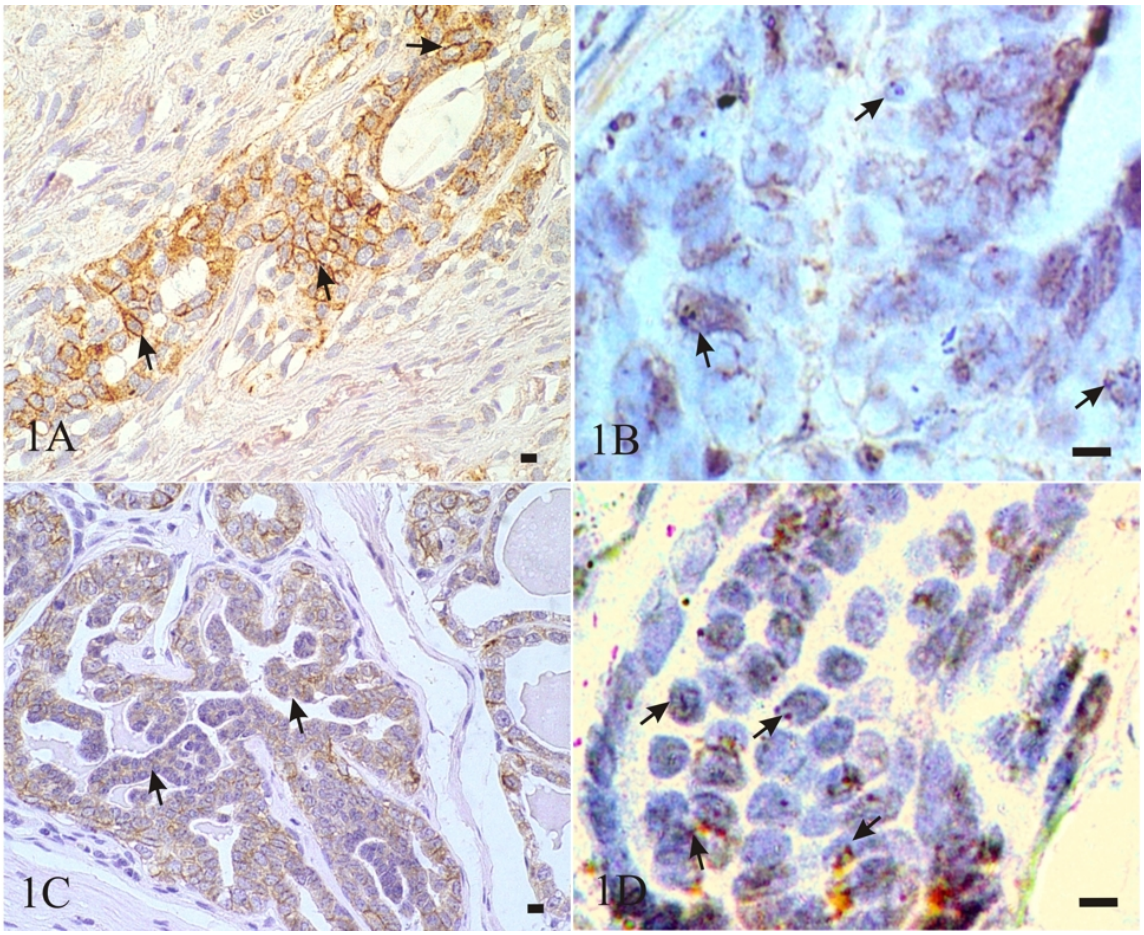

Figure 1: Canine mammary gland. 1A) Carcinoma in a benign mixed tumor, 600x; Immunohistochemistry: malignant epithelial component shows intense membrane staining for HER2 in more than 30% of tumor cells (3+ score); 1B) Carcinoma in a benign mixed tumor, 1000X; Chromogenic in situ hybridization: HER-2 oncogene copies in invasive mammary carcinoma cells. One or two brown dots per nucleus are seen in both malignant epithelial cells (arrows); 1C) Atypical ductal hyperplasia, 600x; Immunohistochemistry: atypical ductal hyperplasia showing negative membrane expression of HER2 in epithelial cells; 1D) Atypical ductal hyperplasia; 1000X; Chromogenic in situ hybridization: the normal HER2 gene appears as one or two gene copies in atypical ductal cells hyperplasia (arrows). Bar = 10 μm.

7. CONSIDERAÇÕES FINAIS

A compreensão do comportamento das alterações mamárias caninas auxilia, cada vez mais, na escolha da intervenção veterinária a ser tomada junto ao paciente oncológico. Aliado a isso trabalhos atuais demonstram que, na procura de um modelo animal mais adequado em pesquisas sobre patologia mamária comparada, a cadela apresenta características patológicas que mais se assemelham com seres humanos. A partir dessa proposta e de acordo com os resultados encontrados no presente estudo, observamos que as mudanças no comportamento celular que provavelmente são responsáveis pelo surgimento de carcinomas mamários invasivos em na espécie humana também podem ser identificadas na glândula mamária canina.

As lesões epiteliais não invasivas presentes na mama canina, sejam elas neoplásicas ou não neoplásicas, apresentam comportamento morfológico e expressão molecular indicativos de transformação maligna. Identificar quais são as lesões envolvidas nesse processo de transformação ainda é um desafio. O que podemos relatar, a partir dos resultados apresentados é que existem diferentes tipos de lesões epiteliais, algumas ainda pouco conhecidas, como as lesões de células colunares, e outras já bem relatadas como as hiperplasias ductais. E essas lesões epiteliais estão frequentemente associadas a neoplasias mamárias caninas. Aliado a isso, observa-se que o comportamento das lesões intraepiteliais não neoplásicas, indica um possível acúmulo de modificações moleculares, que podem culminar com o surgimento de carcinomas mamários.

É bem conhecido que o surgimento do câncer está fundamentalmente associado ao acúmulo de mutações gênicas que culminam com a perda no controle proliferativo e com a morte

celular, incluindo modificações moleculares associadas ao comportamento funcional da célula (diferenciação celular). Dentre as lesões estudadas, aquelas que apresentam alto grau de atipia celular (hiperplasias ductais atípicas e lesões de células colunares com atipia) possuem esse tipo de comportamento, que pode ser idenficado também nos carcinomas *in situ* e invasores que estão presentes na mesma glândula mamária acometida. A presença dessas alterações na mama canina, além de possuir um importante papel, ao auxiliar na compreensão da carcinogênese mamária, também deve ser vista como um fato novo, que pode fornecer informações relevantes na escolha da conduta clínica a ser tomada junto ao paciente, nesse caso o cão.

É conhecida a elevação do risco de surgimento de uma nova lesão neoplásica, quando diagnosticado inicialmente uma lesão hiperplásica na glândula mamária humana. Esse fato ainda é pouco estabelecido em medicina veterinária e não existem trabalhos que mostram uma relação entre o diagnóstico inicial de hiperplasias mamárias e o surgimento posterior de uma lesão neoplásica. Propostas de trabalhos prospectivos poderiam esclarecer esse questionamento, entretanto as condutas atuais em relação à escolha do tratamento de tumores mamários caninos, devido ao momento tardio do diagnóstico, ainda não permitem esse tipo de análise. O diagnóstico de tumores mamários muito tardiamente, quando se apresentam como grandes massas, leva a retirada da mama acometida, de um segmento ou de toda a cadeia, o que invalidaria esse tipo de análise.

Novas propostas relacionadas a identificação de modificações no conteúdo genético e na determinação de clonalidade celular também poderão auxiliar na compreensão da carcinogênese mamária canina. Estudos com perspectivas promissoras foram realizados por nosso grupo previamente e permitiram sugerir origem monoclonal em neoplasias mamárias caninas, podendo provavelmente tornar-se foco de novos estudos no futuro.

8. CONCLUSÕES

- Dentre as lesões intraepiteliais não neoplásicas caninas, foram identificadas hiperplasias ductais (com e sem atipia) e descritas pela primeira vez às alterações de células colunares. Essas lesões apresentam comportamento morfológico semelhante ao descrito na classificação de lesões intraepiteliais mamárias humanas.

- As lesões mais frequentemente observadas na mama canina são as hiperplasias ductais sem atipia celular, seguido de hiperplasias ductais atípicas e alterações de células colunares. Sendo indentificada uma relação direta entre a presença de lesões atípicas (hiperplasias e alterações de células colunares) associadas à presença de carcinomas *in situ* e invasores da mama.

- O comportamento imuno-fenotípico das hiperplasias ductais sugere que essa alteração possa representar uma etapa nos processos alterativos observados na carcinogênese mamária. Sendo identificada maior expressão para o marcador de proliferação celular Ki-67 e receptores para fator de crescimento epitelial (EGRF), perda de expressão de receptores hormonais (RE e RP), E-caderina e citoqueratina 34βe-12 e ausência de marcação para HER2. Em lesões atípicas esse comportamento mostra-se mais evidente, assemelhando-se ao comportamento observado em carcinomas *in situ* e invasores.

- Semelhante ao descrito para as alterações de células colunares na glândula mamária humana, foram observados comportamentos imuno-fenotípicos na glândula mamária canina, sugestivos de transformação neoplásica, com elevada expressão de proteínas envolvidas no ciclo celular (Ki67, RE, RP, EGRF), com maior evidência na presença de atipia celular.

- Não foram identificadas amplificações do gene HER2 em carcinomas invasores HER2 positivos e nas hiperplasias atípicas associadas a esses tumores na glândula mamária canina.

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10 . ANEXOS

Anexo I- Carta de aprovação do COEP/UFMG - Novembro 2006

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 192/2006,** relativo ao projeto intitulado "***Caracterização histomorfológica e imuno-histoquímica das hiperplasias epiteliais mamárias em fêmeas da espécie canina***", que tem como responsável **Geovanni Dantas Cassali** , está de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **7/ 03/2007.**

Este certificado expira-se em **7/ 03 / 2012.**

CERTIFICATE

We hereby certify that the **Protocol nº 192/2006,** related to the project entitled "***Histomorfological and immunoshistochemistry caracterization of the mammary epithelial hyperplasias in female dogs***", under the supervision of **Geovanni Dantas Cassali,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **March 7, 2007.**

This certificate expires in **March 7, 2012.**

Belo Horizonte, 7 de Março de 2007.

Prof. Humberto Pereira Oliveira
Presidente do CETEA/UFMG

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www.ufmg.br/bioetica/cetea - cetea@prpq.ufmg.br

(Mod.Cert. v1.0)

Anexo II- Protocolo de exame anatomopatologico das lesões mamárias caninas adaptado de Ferreira *et al.* (2003).

Department of General Pathology -*ICB*-
Department Clinica and Cirurgia Veterinary -*EV*

MAMMARY GLAND HISTOLOGICAL EXAMINATION REQUEST FORM

Name: Age: Species: Race:

Clinical Card: Owner: Phone:

Address.:

Petitioner:

Estrous Cycle: 1 Regular 2 Irregular 3 Don't exhibit

Number of delivery: 1 No one 2 One 3 Two 4 Three 5 Others values ( )

Pseudogestation: 1 Yes 2 No

Abortion: 1 Yes 2 No **Castration**: 1 Yes (Date: / /) 2 No

Uterine alterations (secretion)/ When? 1 Yes (Date: / /) 2 No

Has ever matted and not get pregnant? 1 Yes 2 No

Hormone use: 1 contraceptives 2 abortive 3 No

(Type:) (Date / /) How many times? ( )

Development of lesions: When**: (/ /)**

Previous tumoral lesions: 1 Yes 2 No Localization ( ) Surgery:1 Yes 2 No

The Mammary Anatomopathological Protocol

Clinical Examination

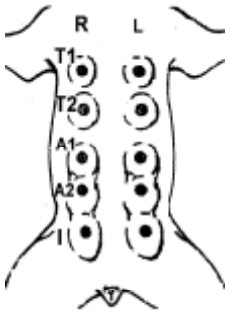
Type of specimen: 1 Biopsy 2 Tumorectomy 3 Mastectomy 4 Others:_____

Dimension of the part: 1)_ _ _ x _ _ _ x _ _ _ cm 2)_ _ _ x _ _ _ x _ _ _ cm 3) _ _ _ x _ _ _ x _ _ _ cm

Weight: 1)_ _ _ _ , _ _ _ g 2) _ _ _ _ , _ _ _ g 3) _ _ _ _ , _ _ _ g

Mammary Gland(s)	Consistency	Tumor	Nipple	Secretion	Ulceration	Lymph nodes involved	Characterization of the material	Cytological examination
T_1r, T_1l, T_2r, T_2l, A_1r, A_1l, A_2r, A_2l, Ir, Il	Firm(1) Soft(2) Elastic (3)	No fixed(1) Fixed to skin(2) Fixed to muscle.(3)	Without alteration(1) Ulceration(2) Retraction(3) Eczema(4) Absent(5)	Yes (1) No (2)	Yes (1) No (2)	Axilar (1) Inguinal (2) SAA (3)	Solid lesion (1) Cystic lesion(2) Infiltration (3)	Insufficient material (1) Benign (2) Malignant (3) Not done (4)

Mammary Gland (s)



Date: ____/___/_____

_____

(Clinical/Pathologist)

Anexo II- Protocolo de exame anatomopatologico das lesões mamárias caninas adaptado de Ferreira *et al.* (2003). *continuação.*

Histopathological Examination

Identification of the tumor Localization: _____ (T_1r, T_2r, A_1r,.etc.) Size:_ _ _ x _ _ _ x _ _ _ cm

Classification: 1 Normal 2 Benign Lesions 3 Benign Neo 4 Malignant Neo 5 Without residual tumor

Benign Lesions: 1 Adenosis 2 Duct ectasia 3 Focal fibrosis 4 Cysts 5 Others:_____

Epithelial Hyperplasia: 1 Absent 2 Present

Benign Neoplasia:

1 Fibroadenoma 2 Simple adenoma 3 Complex adenoma 4 Basaloid adenoma

5 Benign mixed tumor 6 Duct papilloma 7 Others:_____

Malignant Neoplasia:

Noninfiltranting " in situ" carcinoma: 1 Yes 2 No

Mioepitelial Involvement: 1 Simple 2 Complex

Infiltrating (Histological classification):

1 Papillary carninoma 2 Tubular carcinoma 3 Solid carcinoma 4 Anaplastic carcinoma

5 Spindle cell carcinoma 6 Squamous carcinoma 7 Mucinous carcinoma 8 Others:_____

Tubular arrangement: 1 (+75%) 2 (25-75%) 3 (- de 25%)

Cytological degree (pleomorphism): 1 Few 2 Moderate 3 Numerous Ones

Mitosis: 1 Few (1 hpf) 2 Moderate (2 hpf) 3 Numerous Ones (3 hpf)

Histological degree: 1 I well 2 II moderate 3 III poorly

Infiltration of the skin: 1 Absent 2 Present 3 Lymphatic embolism 4 It is not applied

Muscular invasion: 1 Absent 2 Present 3 Not applied

Invasion of peritumoral lymphatic vessels: 1 Yes 2 No

Invasion of peritumoral blood vessels: 1 Yes 2 No

Necrosis: 1 Absent 2 Discrete 3 Moderate 4 Intense

Lymphocytic infiltrate: 1 Absent 2 Discrete 3 Moderate 4 Intense

Estromal Desmoplasia: 1 Absentee 2 Discrete 3 Moderate 4 Intense

Elastosis: 1 Absentee 2 Discrete 3 Moderate 4 Intense

Microcalcifications: 1 Absent 2 Moderate 3 Numerous Ones

Surgical edge: 1 Absent 2 Present (_____ mm)

Nipple: 1 Free 2 Intracanalicular infiltration 3 Estromal infiltration 4 Not applied

Soft tissue: 1 Without alterations 2 Benign lesions

Lymph nodes: (1) Without metastasis (2) Metastasis 1 Inguinal ( ) 2 Axilar ( ) 3 Others ( )

Infiltration of the capsule: 1 Yes 2 No

Sinusal histiocitosis: 1 Absent 2 Present

Pathological staging: pT____ pN___ pM___

Comments: _____

Conclusion:_____

Date: ____/___/_____ _____

(Pathologist)

Anexo III – Produção científica relacionada à tese no período de Fevereiro de 2007 a Março de 2010:

Artigo Publicado:

FERREIRA, E; GOBBI, H; SARAIVA, BS; CASSALI, GD. Columnar cell lesions of the canine mammary gland: pathological features and immunophenotypic analysis. BMC Cancer (Online), v. 10, p. 61, 2010.

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Anexo IV - Produção científica não relacionada à tese no período de Fevereiro de 2007 a Março de 2010

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FERREIRA, E; SILVA, AES; SERAKIDES, R; GOMES, AS ; CASSALI, GD. Model of induction of thyroid dysfunctions in adult female mice.. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, v. 59, p. 1245-1249, 2007.

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