



Histopathological and immunohistochemical assessment of invasive micropapillary mammary carcinoma in dogs: A retrospective study

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ABSTRACT

Invasive micropapillary carcinoma (IMPC) of the mammary gland, despite its rare occurrence in humans and dogs, is an important neoplasm due to its aggressive behaviour. The aim of this study was to evaluate the clinicopathological and immunophenotypical characteristics of IMPC and to determine the overall survival of dogs with this tumour. Of the selected cases, the majority had >3 cm neoplasms (15/19, 78.95%) and lymph node metastases (16/16, 100%), but only two cases (2/9, 22.2%) had distant metastases.

The IMPCs were classified as either pure (15/22, 68.18%) or mixed (7/22, 31.82%) types. There was a predominance of moderate histological grade tumours (16 grade II) and the average overall survival was 120 days. Positive immunohistochemical staining for epithelial membrane antigen and negative staining for CD-31, p63 and cytokeratin (CK) AE1/AE3 in cystic formations confirmed the micropapillary nature of these neoplasms. A proportion of cases exhibited positive epithelial staining for p63 (4/20, 20%) and CK34βE12 (20/22, 90.9%). Most cases were positive for oestrogen (19/20, 95%) and progesterone (19/20, 95%) receptors, but lacked HER-2 (16/22, 72.72%) and epidermal growth factor receptor (15/22, 68.18%) over-expression. The mean proliferation index was 14.8%. The findings demonstrate that, similar to humans, canine IMPCs behave aggressively with high rates of metastasis to regional lymph nodes and short overall survival times.

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Introduction

Invasive micropapillary carcinoma (IMPC) of the breast is a morphologically distinct form of invasive ductal carcinoma found in women (Nassar et al., 2004). The constituent tumour cells are typically arranged in small clusters with a central lumen and micropapillae lacking fibrovascular cores extending into clear spaces. The tumour is highly malignant with a high propensity for vascular invasion and lymph node metastases (Luna-Moré et al., 1994). The definitive diagnosis of IMPC relies on the use of immunohistochemical (IHC) markers such as epithelial membrane antigen (EMA), CD-31 and cytokeratin (CK) AE1/AE3 (Nassar et al., 2004; Pettinato et al., 2004).

In dogs, there have only been three previous reports of this tumour which demonstrates many similarities to the human neoplasm, such as a high incidence of lymph node metastasis, high histological grade, and low overall survival rates (Cassali et al., 1999, 2002; Gama et al., 2008). Based on these findings, IMPC has been included as an independent subtype in recently published

histological classification systems of canine mammary tumours (Cassali et al., 2011; Goldschmidt et al., 2011).

Several clinicopathological features have been used in determining a prognosis for canine mammary tumours. Poor prognostic indicators include tumours >5 cm, lymph node metastasis, and high histological grade (Karayannopoulou et al., 2005; Ferreira et al., 2009). Hormone receptor negativity, over-expression of human epidermal growth factor receptor (HER)-2 and epidermal growth factor receptor (EGFR), and a high proliferation index are also frequently associated with aggressive behaviour in mammary neoplasms (Peña et al., 1998; Geraldles et al., 2000; Dutra et al., 2004; Bertagnolli et al., 2011). Given the paucity of studies evaluating the immunophenotypical features and prognosis of canine IMPC, the objective of this study was to describe the clinicopathological and immunophenotypical characteristics of canine IMPC, and to determine the overall survival rates of dogs diagnosed with this tumour.

Materials and methods

Case selection and evaluation

All procedures were performed under the appropriate guidelines and with the approval of the Ethics Committee for Animal Experimentation (CETEA/UFMG, protocol 211/2009).

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Twenty-two cases of canine IMPC, based on histopathological assessment, were selected from the Veterinary School of the Federal University of Minas Gerais, the Federal University of Bahia, and the Laboratory of Comparative Pathology at the Institute of Biological Sciences at the Federal University of Minas Gerais. The cases were staged according to the tumour-node-metastasis (TNM) clinical staging system for canine mammary tumours: this system evaluates tumour size (T_1 , 0–3 cm; T_2 , 3–5 cm; T_3 , >5 cm); involvement of regional lymph nodes (N_0 , no metastasis; N_1 , metastasis), and the presence of distant metastasis (M_0 , no metastasis; M_1 , metastasis). Cases are then categorised into five stages: I ($T_1N_0M_0$); II ($T_2N_0M_0$); III ($T_3N_0M_0$); IV ($T_{1,2,3}N_1M_0$); and V ($T_{1,2,3}N_{0,1}M_1$) (Owen, 1980).

Tumour specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and 4 μ m thick histological sections were cut and stained with haematoxylin and eosin. All cases were reviewed and re-classified independently by two veterinary pathologists (COG and GDC). The IMPCs were classified according to the WHO human histological classification criteria (Tavassoli and Devillee, 2003), and any associated mammary lesions were classified by the criteria of Misdorp et al. (1999). We defined 'pure' IMPC as carcinomas with a >75% infiltrating micropapillary pattern, and 'mixed' IMPC as carcinomas with a <75% infiltrating micropapillary pattern associated with other infiltrating carcinomas (Middleton et al., 1999; Zekioglu et al., 2004). In situ regions of micropapillary carcinoma, vascular emboli, and metastasis to regional lymph nodes were

evaluated. In situ micropapillary areas were classified as low, intermediate and high nuclear grade according defined criteria (Consensus Conference Committee, 1997). The tumour was graded according to the Nottingham Grading System (Elston and Ellis, 1991, 1998).

Overall survival time was defined (in days) as the period between surgery and death due to the tumour. The follow-up period was 330 days. Animals that died from unknown causes or causes unrelated to the tumour were censored. The survival rate was calculated using the Kaplan–Meier method, and statistical significance was examined using a log-rank test where $P < 0.05$ was considered significant.

Immunohistochemical examinations

Consecutive 5 μ m-thick sections were mounted on silanised slides, and a biotin-peroxidase system was used with secondary antibodies identified using 'Advance HRP' (Dako North America). Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution in methyl alcohol. Reagents were applied manually and immunoreactivity was visualised by incubating the slides for 10 min with diaminobenzidine (DAB Substrate System, Laboratory Vision). Details of the antibodies, dilutions, antigen retrieval procedures, and incubation times used are given in Table 1. Sections from a HER-2, EGFR, oestrogen receptor (OR),

Table 1
Details of immunohistochemical reagents and methods used in the study. AR, antigen retrieval; OR, oestrogen receptor; PR, progesterone receptor; EMA, epithelial membrane antigen; EGFR, epithelial growth factor receptor.

Target antigen	Clone	Manufacturer	Dilution	AR method	Incubation time (h)
OR	1D5	Dako	1:20	Pressurised heating (125 °C)	1
PR	HPRA2	Neomarkers	1:20	Pressurised heating (125 °C)	1
EMA	E29	Dako	1:25	Water bath (98 °C)	16
Ki-67	Mib-1	Dako	1:25	Pressurised heating (125 °C)	1
p63	4a4	Neomarkers	1:100	Water bath (98 °C)	16
Cytokeratin	AE1/AE3	Dako	1:100	Water bath (98 °C)	1
CD-31	JC70A	Dako	1:20	Water bath (98 °C)	16
Cytokeratin	34 β E12	Dako	1:50	Water bath (98 °C)	1
EGFR	31G7	Zymed	1:100	Water bath (98 °C)	16
C-erb B2	Polyclonal	Dako	1:200	Water bath (98 °C)	16

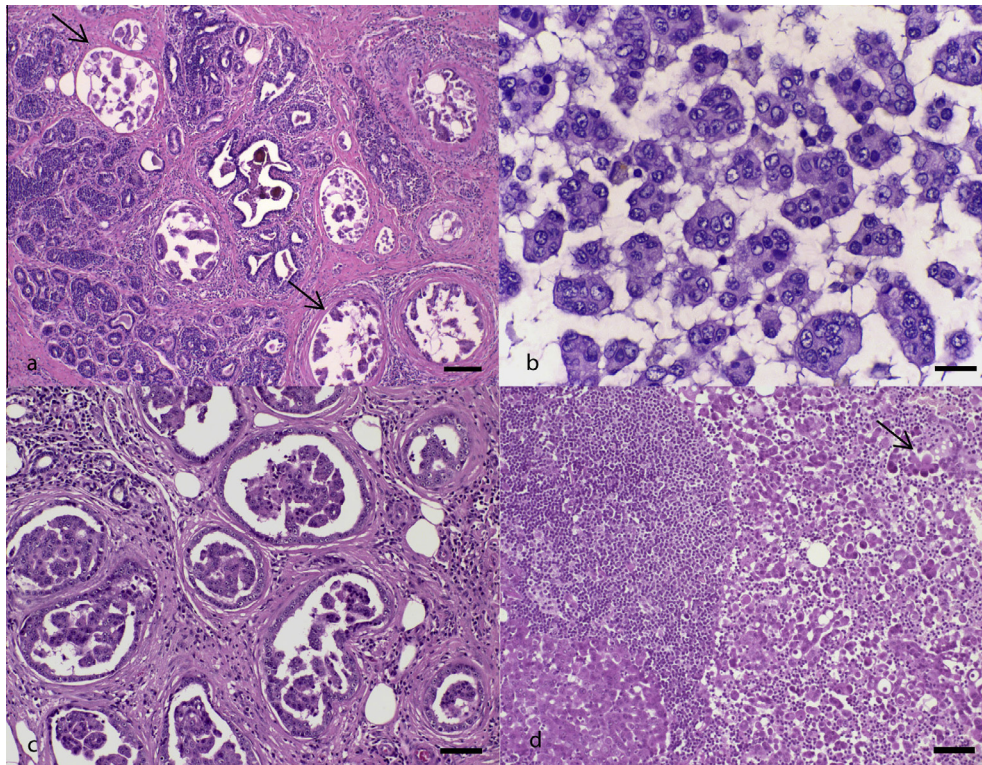


Fig. 1. Photomicrographs illustrating features of invasive micropapillary carcinoma of the canine mammary gland: (a) invasive micropapillary areas characterised by neoplastic epithelial cells within cystic spaces (arrow) (HE stain, scale bar, 90 μ m); (b) moderately pleomorphic neoplastic epithelial cells within cystic spaces (HE stain, scale bar, 20 μ m); (c) in situ micropapillary carcinoma (HE stain, scale bar, 50 μ m); (d) lymph node metastasis with similar cellular arrangement to that of primary tumour (arrow) (HE stain, scale bar, 50 μ m).

progesterone receptor (PR), EMA, CK AE1AE3, CK34βE12, MIB-1 and p63 positive canine mammary carcinoma were used as a positive control. For the CD-31 marker, normal blood vessels within the tumour were used as an internal positive control. Negative controls were assessed using normal serum (Ultra V Block, Laboratory Vision) as the primary antibody.

HER-2 and EGFR expression were determined by cell membrane staining and scored according to the guidelines established by the American Society of Clinical Oncology, College of American Pathologists (ASCO/CAP) (Wolff et al., 2007). Staining for OR and PR was evaluated, and cases scored positive if nuclear staining was present in ≥1% of the tumour cells (Hammond et al., 2010). The proliferative index was calculated by counting the number of nuclei positive for Ki-67 (anti-MIB-1) staining in a total of 1000 neoplastic cells from each lesion (Dutra et al., 2008). Strong immunoreactivity of CK34βE12, regardless of the extent, and moderate-to-weak expression in >10% of the total tumour area was defined as positive (Yamaguchi et al., 2010). The presence or absence of other markers (CKAE1AE3, CD-31 and p63) was also evaluated.

Results

The ages of the animals at the time of surgery ranged from 7 to 13 years (mean 10.95 years ± 1.80). Multi-centric localisation was predominantly observed (12/20, 60%) in relation to the inguinal (6/20, 30%), cranial thoracic (1/20, 5%), and caudal abdominal (1/20, 5%) regions. In two remaining cases, localisation was not confirmed. Tumour size was evaluated in 19 cases and classified as T₁ (4/19, 21%), T₂ (5/19, 26.3%), or T₃ (10/19, 52.6%). The status of the regional lymph node was evaluated in 16 cases, with 100% demonstrating metastasis. Only 2/9 (22.2%) cases were positive for distant metastases. Spread to the iliac lymph node and lung were observed in mixed and pure IMPCs, respectively. Clinical staging was possible in only six cases, with 100% classified as stage IV.

All IMPC cases demonstrated numerous irregular stromal cystic formations containing nests of epithelial cells in a morula pattern (Fig. 1a). In a few tumours, a lumen was present within the cell clusters. Neoplastic epithelial cells were pleomorphic with typically polygonal outlines and eosinophilic cytoplasm (Fig. 1b). Tumours exhibited pure (15/22, 68.2%) and mixed (7/22, 31.8%) micropapillary patterns (papillary carcinoma, 4/7, 57.1%; carcinomas arising from benign mixed tumours, 2/7, 28.6%; carcinosarcoma, 1/7, 14.3%). The majority of cases were of moderate (grade II, 16/22, 72.7%) as opposed to high (grade III, 3/22, 13.6%) or low (grade I, 3/22, 13.6%) grade. In situ micropapillary carcinoma was associated with infiltrating carcinomas in 14 cases (63.6%) (Fig. 1c) and showed intermediate (8/14; 57.1%) and high (6/14; 42.9%) nuclear grades. All 16 lymph nodes evaluated contained metastatic foci exhibiting micropapillary (13/16, 81.2%) (Fig. 1d), or mixed (micropapillary and other patterns) (3/16, 18.8%) tumour patterns. Neoplastic emboli in the micropapillary pattern were also observed (19/22, 86.4%). The clinicopathological features of pure and mixed IMPC are summarised in Table 2.

Survival data were available for 17 cases, and 14 dogs (82.3%) died as a consequence of mammary neoplasia – 2/14 were euthanased due to the disease. Eleven of these 14 dogs presented only with regional metastasis; one animal had both regional and distant metastases, and for two dogs this information was not available. One dog (5.9%) died from haemorrhagic diathesis 8 days after surgery, and two dogs (11.8%) were alive 30 days post-surgery. The median overall survival time was 120 days. Although a relatively small number of cases were evaluated, the median overall survival time for cases of pure IMPC (120 days) was shorter than that for the mixed type (180 days) ($P = 0.62$).

The results of the immunohistochemical examinations for HER-2, EGFR, OR, PR, CKAE1AE3, CK34βE12, p63 and EMA are detailed in Table 3. All cases demonstrated intense cytoplasmic staining for cytokeratin AE1/AE3. Immunohistochemical labelling for EMA was particularly intense on the stromal (basal) aspect of the cell clusters, which accentuated the outlines of the micropapillary

Table 2

Details of the clinicopathological features of 'pure' compared with 'mixed' canine mammary invasive micropapillary carcinoma (IMPC).

	Pure IMPC (n = 15)	Mixed IMPC (n = 7)
Mean age (years) (n = 21)	11.4 ± 1.8	9.8 ± 1.2
Median tumour size (cm; maximum–minimum) (n = 19)	5.11 (10–0.5)	7.5 (13–4)
<i>Breed (n [%]) (n = 20)</i>		
Crossbreed	3(21.4)	3(50)
Cocker	3(21.4)	0(0)
Poodle	2 (14.3)	1(16.7)
Other breeds ^a	6 (42.9)	2 (33.3)
<i>Cutaneous ulceration</i>		
Absent	3 (20)	2 (28.6)
Present	12 (80)	5 (71.4)
<i>Histological grade (n [%])</i>		
I	5(33.3)	1(14.3)
II	7(46.7)	6(85.7)
III	3(20)	0(0)
<i>Stage (TNM; n [%])(n = 6)</i>		
I	0 (0)	0 (0)
II	0 (0)	0 (0)
III	0 (0)	0 (0)
IV	4 (100)	2 (100)
V	0 (0)	0 (0)
<i>In situ micropapillary carcinoma</i>		
Absent	7(46.7)	1 (14.3)
Present	8(53.3)	6(85.7)
<i>Vessel invasion (n [%])</i>		
Absent	4 (26.7)	0(0)
Present	11(73.3)	7(100)
<i>Lymph node metastasis (n = 17)</i>		
Absent	0(0)	0(0)
Present	12(100)	5(100)
<i>Distant metastasis (n = 9)</i>		
Absent	4 (80)	3(75)
Present	1 (20)	1(25)

^a Breeds with only one case each: Bichon Frise, Dachshund, Dalmatian, Doberman, Siberian Husky, Akita, Pinscher and Labrador Retriever.

Table 3

Immunohistochemical analysis of the following tissue markers in canine mammary invasive micropapillary carcinoma: human epidermal growth factor receptor (HER)-2, epidermal growth factor receptor (EGFR), oestrogen receptor (OR), progesterone receptor (PR), cytokeratin (CK)AE1AE3, CK34βE12, p63 and epithelial membrane antigen (EMA).

Antibody	Number of dogs (%)			
	0	+	++	+++
OR ^a	1/20 (5)	19/20 (95)	NA	NA
PR ^a	1/20 (5)	19/20 (95)	NA	NA
HER-2 ^b	8/22 (36.4)	8/22 (36.4)	3/22 (13.6)	3/22 (13.6)
EGFR ^b	1/22 (4.6)	14/22 (63.6)	3/22 (13.6)	4/22 (18.2)
p63 ^c	16/20 (80)	4/20 (20)	NA	NA
CK34βE12 ^d	2/22 (9.1)	20/22 (90.9)	NA	NA
CKAE1AE3 ^e	0/22 (0)	22/22 (100)	NA	NA
EMA ^f	0/22 (0)	22/22 (100)	NA	NA

NA, Not applied.

^a '0', <1% nuclear staining in tumour cells; '+', >1% nuclear staining in tumour cells.

^b '0', no staining; '+', weak, incomplete membrane staining of any proportion of tumour cells; '++', complete membrane staining that is either non-uniform or weak in intensity, but with obvious circumferential distribution in ≥10% of cells, or intense, complete membrane staining of <30% of tumour cells; '+++', uniform, intense membrane staining of >30% of tumour cells.

^c '0', no staining; '+', nuclear-positive p63 protein staining in epithelial 'nests' localised within cystic spaces.

^d '0', no staining; '+', strong immunoreactivity, regardless of extent, and moderate-to-weak expression in >10% of total tumour area.

^e '0', no staining; '+', cytoplasmic staining in epithelial cells.

^f '0', no staining; '+', positive staining on stromal aspect of neoplastic clusters.

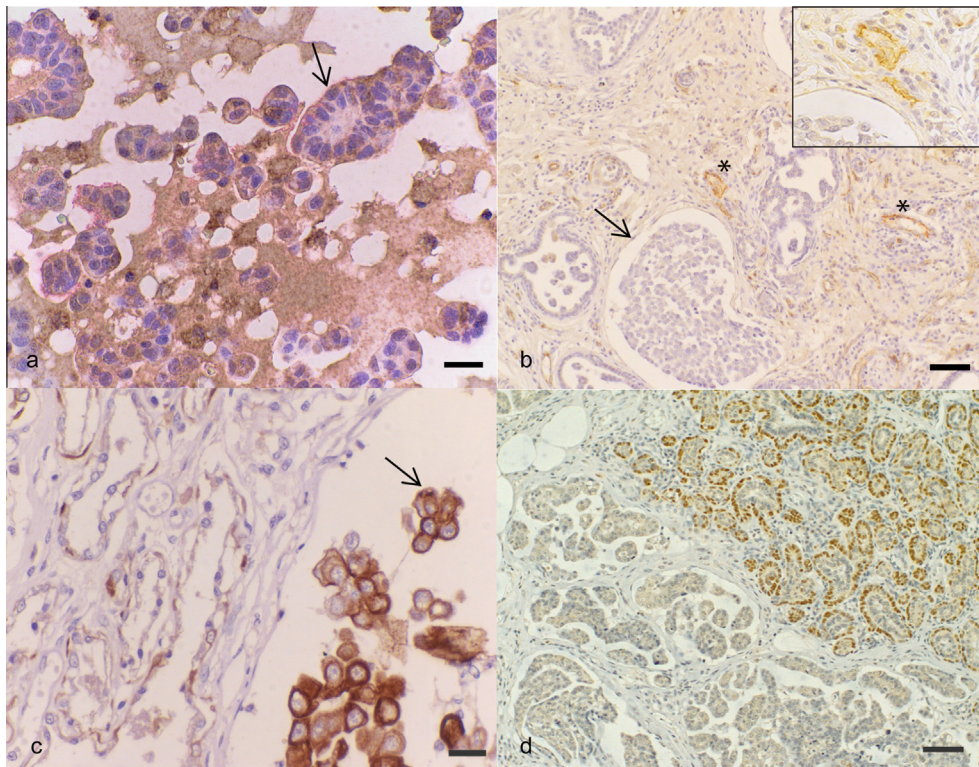


Fig. 2. Photomicrographs illustrating features of invasive micropapillary carcinoma of the canine mammary gland: (a) micropapillae covered by a line of epithelial membrane antigen (EMA) (arrow) (streptavidin–biotin–peroxidase anti-EMA, counterstained with Harris's haematoxylin, scale bar, 20 μ m). (b) Absence of CD31 antigen labelling in the cystic spaces of invasive micropapillary areas (arrow). Blood vessels were positive for CD31 (asterisk) (Inset shows detail) (streptavidin–biotin–peroxidase anti-CD31, counterstained with Harris's haematoxylin, scale bar, 20 μ m). (c) Neoplastic epithelial cells exhibiting CK34 β E12-positive cytoplasm (arrow) (streptavidin–biotin–peroxidase anti-CK34 β E12, counterstained with Harris's haematoxylin, scale bar, 20 μ m). (d) Absence of p63 labelling in the cystic spaces of invasive micropapillary areas (lower left aspect of image) (streptavidin–biotin–peroxidase anti-p63, counterstained with Harris's haematoxylin, scale bar, 50 μ m).

units by forming a distinct band on this surface (Fig. 2a). Positive staining for CD31 was observed in the cytoplasm of endothelial cells of the pre-existing vascular spaces in the region around the tumour, and was not observed in the cystic formations (Fig. 2b). A majority of samples were positive for CK34 β E12 (20/22, 90.9%) (Fig. 2c).

HER-2 (16/22, 72.7%) and EGFR (15/22, 68.2%) were not over-expressed in most IMPCs. Immunostaining for p63, Mib-1, OR and PR was performed in only 20 cases. Staining for p63 on the walls of cystic formations was absent – a feature which differed from the normal tissue, and reflected invasion by the tumour (Fig. 2d). Positive nuclear p63 staining in epithelial nests localised within the cystic spaces was observed in four cases (20%). There

was a predominance of positive staining for the OR (19/20, 95%) (Fig. 3a) and PR (19/20, 95%) (Fig. 3b) markers. The proliferation index determined from the amount of nuclear staining for Mib-1 ranged from 1.7% to 65.9% (mean, 14.8 \pm 14.1%).

Discussion

IMPCs of the mammary gland are extremely aggressive tumours in humans (Luna-Moré et al., 1994) and, based on the results of this and previous studies (Cassali et al., 1999, 2002; Gama et al., 2008), appear to exhibit similar behaviour in dogs. Despite widely varying tumour sizes in both humans and dogs (Siriaunkgul and Tavassoli,

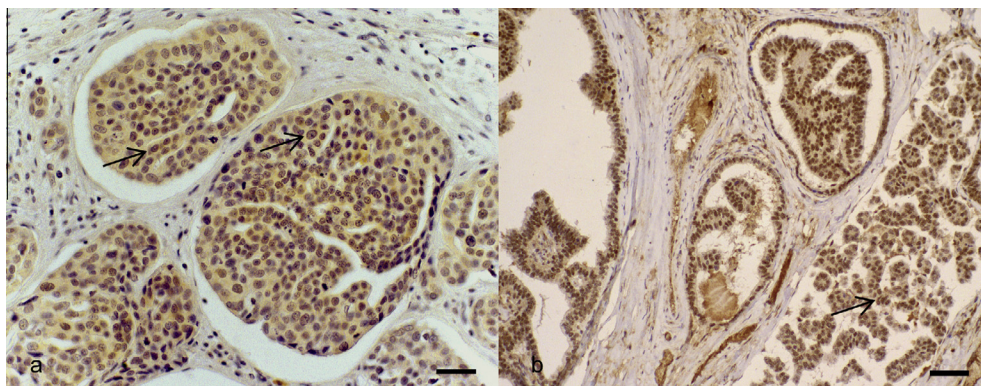


Fig. 3. Photomicrographs illustrating features of invasive micropapillary carcinoma of the canine mammary gland: (a) nuclei of neoplastic, oestrogen receptor (OR)-immunoreactive epithelial cells stain brown (arrows) (streptavidin–biotin–peroxidase anti-OR, counterstained with Harris's haematoxylin, scale bar, 30 μ m); (b) nuclei of neoplastic, progesterone receptor (PR)-immunoreactive epithelial cells stain brown (arrow) (streptavidin–biotin–peroxidase anti-PR, counterstained with Harris's haematoxylin, scale bar, 50 μ m).

1993; Kuroda et al., 2004; Pettinato et al., 2004; Zekioglu et al., 2004; Gama et al., 2008), lymph node metastases are frequently observed in both species (Walsh and Bleiweiss, 2001; Gama et al., 2008), and in the current study we found lymph node metastases in all of the cases evaluated, including T₁ cases.

Moderate grade IMPCs dominated our study, which contrasts somewhat with the preponderance of high grade tumours of this type described in humans (Walsh and Bleiweiss, 2001; Marinho et al., 2007). In the present study, *in situ* micropapillary areas were associated with tumour invasion. In IMPC cases in humans, the intra-ductal component that accompanies these tumours often has a micropapillary pattern and typically has intermediate- to high-grade nuclei. In contrast, more common low-grade nuclear intra-ductal micropapillary carcinomas are generally associated with well differentiated invasive ductal carcinomas (Walsh and Bleiweiss, 2001).

The 2003 WHO histological classification of human breast tumours does not specify the degree of micropapillary invasion required to make a diagnosis of IMPC, and, to date, no consensus has been reached on this issue (Luna-Moré et al., 1994; Middleton et al., 1999; Amendeira et al., 2003; Zekioglu et al., 2004). In the present study, the mixed subtypes of tumour, even when combined with tumours with a better prognosis (such as carcinoma arising from benign mixed tumours and papillary carcinomas) carried a poor prognosis similar to the pure subtype with lymph node metastases and a shorter overall survival.

All of the cases included in our study were positive for both the EMA and CKAE1AE3 markers. The empty spaces surrounding the micropapillae of the carcinoma cell nests were not surrounded by endothelial, myoepithelial cells, or epithelial cells, thus confirming the infiltrative micropapillary nature of the tumour. High rates of CK34βE12-positive staining were observed as described in human IMPCs (Yamaguchi et al., 2010), where CK34βE12 is considered a marker of ductal histogenesis (Gown and Vogel, 1984). This finding thus supports a ductal origin for canine IMPCs.

Nuclear staining for p63 in human IMPC was explained by the concurrence of squamous metaplasia (Yamaguchi et al., 2010). In our study, p63-positive samples demonstrated features typical of squamous metaplasia and CK34βE12-positivity. High rates of positivity for hormonal receptors have been described in human IMPC, and were also observed in the present study (Luna-Moré et al., 1994; Walsh and Bleiweiss, 2001; De la Cruz et al., 2004; Pettinato et al., 2004; Weigelt et al., 2008). Hormonal receptor positivity is generally associated with a favourable prognosis and well-differentiated neoplasms; thus, human and canine IMPC seem to differ in their aggressiveness compared with other breast cancers exhibiting these characteristics.

Both EGFR and HER-2/neu are members of the HER growth factor receptor family whose over-expression carries a poor prognosis in humans (Schippinger et al., 2007). Human IMPC is reported to have a low index of HER-2 and EGFR over-expression, similar to the findings in our study (Kim et al., 2005; Marchiò et al., 2008). Based on the median overall survival of 120 days in our report, and the 3 month survival time described by Gama et al. (2008), the median overall survival for IMPC is shorter than that of both canine papillary and solid (median survival time, 426 days) (Hellmén et al., 1993), and metaplastic and ductal (median survival time, 908 days) (Lavalle et al., 2009) carcinomas. The overall survival time in humans with IMPC is reported to be shorter than that for humans with ductal carcinomas regardless of the percentage of micropapillary invasion (De la Cruz et al., 2004).

Conclusions

The results of our study indicate that the morphological and immunohistochemical features of canine IMPC are similar to

findings in humans. Regardless of whether of the pure or mixed subtype, canine IMPC carries a poor prognosis, given their aggressive behaviour, a high tendency to metastasise to regional lymph nodes, and overall shorter survival times.

Conflict of interest statement

None of the authors have any financial or personal relationships that could inappropriately influence or bias the content of this paper.

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