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CLINICOPATHOLOGICAL SIGNIFICANCE OF CASPASE-3 AND KI-67 EXPRESSION IN CANINE MAMMARY GLAND TUMOURS

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Fifty canine mammary gland tumours (CMGT) (18 benign and 32 malignant) were studied by immunohistochemical detection of active caspase-3 and Ki-67 antigens in order to determine their association with several clinicopathological parameters. The percentage of caspase-3 positive cells was significantly higher in benign tumours as compared to their malignant counterparts ($P \leq 0.001$). In the group of malignant tumours there was no significant association between active caspase-3 and the clinicopathological variables considered. The percentage of Ki-67 positive cells was significantly higher in malignant tumours compared to the benign ones ($P \leq 0.001$). In the group of malignant tumours, Ki-67 expression showed a statistically significant association with tumour size ($P = 0.025$), histological type ($P = 0.010$), mitotic grade ($P \leq 0.001$), nuclear grade ($P = 0.025$), differentiation grade ($P = 0.004$), histological grade of malignancy ($P = 0.002$), and presence of metastases in regional lymph nodes ($P = 0.025$). Furthermore, this study revealed a negative correlation between the percentages of active caspase-3 and Ki-67 ($r = –0.39$; $P = 0.04$). Thus, our results suggest a loss of balance between cell death and cell division in CMGT.

Key words: Apoptosis, caspase-3, Ki-67, proliferation, canine mammary gland tumour

One of the best understood mechanisms of programmed cell death is apoptosis, which plays an essential role in the maintenance of homeostasis and development, as well as in cellular stress response (Elmore, 2007; Roth, 2009). Apoptosis is an orchestrated cellular mechanism profoundly implicated in developmental biology and tissue homeostasis, and it occurs under both physiological and pathological conditions (Kim et al., 2006). Some studies have demonstrated

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that dysregulated apoptosis is associated with the development of cancer and contributes to the different steps of carcinogenesis (de Bruin and Medema, 2008). Apoptosis can be triggered by intrinsic and extrinsic pathways and, in mammals, is mediated by a family of cysteine proteases known as caspases (Degterev et al., 2003). Once activated, caspases can cleave a wide variety of cellular targets, such as cytoskeletal proteins, oncoproteins, and proteins of the DNA repair system. Particularly, active caspase-3 is responsible for the fragmentation of DNA (Hadjiloucas et al., 2001). The appearance of this molecule in the cytoplasm of apoptotic cells is an early event that precedes the development of classical apoptotic morphological features. Thus, the immunohistochemical evaluation of active caspase-3 allows the detection of an early stage of apoptosis (Hadjiloucas et al., 2001). In addition, caspase-3 is considered the main executioner of cell-death-dependent caspase activation and an important apoptotic indicator of morphological and biochemical changes associated with apoptosis (Degterev et al., 2003). However, the view of apoptosis as the only form of programmed cell death, entirely dependent on caspases, is now challenged by several findings. Accumulating evidence suggests that cell death can occur in a programmed pattern but in the complete absence, and independently, of caspase activation (Cummings et al., 2004; Tait and Green, 2008). Alternative models of programmed cell death have been proposed, including autophagy, paraptosis, mitotic catastrophe, and caspase-independent cell death (CICD) pathways. All of them are important safeguard mechanisms to protect the organism against unwanted and potentially harmful cells when caspase-mediated pathways fail. Moreover, they can also be triggered in response to cytotoxic agents and cancer (Cummings et al., 2004; Tait and Green, 2008; Constantinou et al., 2009). Although CICD and caspase-dependent cell death have different enzymes involved in their cascades, they often share common characteristics, and sometimes these two pathways can be activated simultaneously by the same stimuli (Cregan et al., 2004).

Programmed cell death, especially apoptosis, has been extensively investigated in a variety of cancers including mammary tumours (Okada and Mak, 2004; Hanahan and Weinberg, 2011). In human breast cancer, there is no consensus regarding the role of apoptosis in carcinogenesis. Some authors claim that high apoptotic indices are related to tumour aggressiveness. However, other researchers found no relationship between the apoptotic index and the parameters of tumour aggressiveness. Regarding canine mammary gland tumours (CMGT), a few studies have been conducted to investigate the role of apoptosis in carcinogenesis and tumour malignancy, using different antibodies (Kumaraguruparan et al., 2006; Yang et al., 2006). A recent study using an antibody against the active caspase-3 has not found a statistically significant correlation with the clinicopathological parameters considered (Bongiovanni et al., 2014).

Uncontrolled proliferation is an already known hallmark of malignancy in human and canine mammary tumours, and several studies have focused on this

topic (Labelle et al., 2004; De Matos et al., 2006; Richardsen et al., 2012; Santos et al., 2013). Ki-67 is a non-histone nuclear protein associated with cellular proliferation and is usually detected with MIB-1 antibody. Although the precise function of this protein is uncertain, its tight association with the cell-cycle regulation phase and its short half-life render this protein a valid marker for the assessment of the growth fraction of neoplastic cells (Klopfleisch et al., 2011; Inwald et al., 2013; Ricciardi et al., 2015). The high Ki-67 index has been positively correlated with metastasis, death from neoplasia, low disease-free survival rates, and low overall survival rates in both humans and dogs (Labelle et al., 2004; De Matos et al., 2006; Richardsen et al., 2012; Santos et al., 2013).

Anomalies in the activity of the regulatory proteins that guarantee the orderly progression of cells can lead to increased cell proliferation, poor maintenance of chromosomal integrity, or promotion of tumour growth (Hanahan and Weinberg, 2011).

As far as we know, there is only a single study on cleaved caspase-3 expression in CMGT (Bongiovanni et al., 2014), and only few studies have investigated the possible relationship between apoptosis and proliferation in these tumours. The goal of the present work was to elucidate the relationship between apoptosis and tumour proliferation in CMGT.

Materials and methods

Tumour specimens

Samples from 50 CMGTs were included in the study. The material was fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE). The tumours were classified according to the World Health Organization (WHO) criteria for CMGTs (Misdorp, 1999) and graded as described by Goldschmidt et al. (2011). Each sample was assessed for size (T1 < 3 cm; T2 ≥ 3 and < 5 cm; T3 ≥ 5 cm), skin ulceration, histological type, presence of necrosis, mitotic grade, nuclear grade, differentiation grade, histological grade of malignancy, and regional lymph node metastasis.

Immunohistochemistry (IHC)

The detection of caspase-3 and Ki-67 was carried out by the streptavidin–biotin–peroxidase complex method, using the Ultra Vision Detection System kit (Lab Vision Corporation) according to the manufacturer's instructions.

For caspase-3 and Ki-67, 3-μm consecutive sections were cut and mounted on silane-coated slides. For caspase-3, antigen retrieval was performed by microwave treatment for 2 × 5 min at 600 W in 0.01 M citrate buffer (pH 6.0), fol-

lowed by cooling for 20 min at room temperature. For Ki-67, antigen retrieval was performed by microwave treatment for 4 × 5 min at 750 W in 0.01 M citrate buffer (pH 6.0), followed by cooling for 20 min at room temperature. All sections were incubated with a primary monoclonal antibody specific for caspase-3 (clone ASP 175, Cell Signaling Technology; 1:200 dilution; 24 h at 4 °C) and for Ki-67 (clone MIB-1, Dako, 1:50 dilution; 24 h at 4 °C). The antibody reaction products were 'visualised' with the chromogen 3,3′-diaminobenzidine tetrachloride (DAB) at 0.05% concentration with 0.01% H_2O_2 (30%). After final washing in distilled water, the sections were counterstained with haematoxylin, dehydrated, cleared and mounted. The primary antibody was replaced with phosphate buffered saline (PBS) for negative controls. Positive controls were the thymus of a young dog for caspase-3 and canine epidermis for Ki-67 (an internal positive control).

Quantification of immunoreactivity

The quantification of immunoreactivity was performed simultaneously by two observers. To evaluate caspase-3 and Ki-67 immunoreactivity, two areas with the most intense labelling were selected, avoiding areas of necrosis or inflammatory cell infiltration. In the selected areas a total of 1000 tumour cells were counted with the help of a microscopic grid, at high magnification. The results were expressed as a percentage of labelled cells in a total of 1000 tumour cells (Pathmanathan and Balleine, 2013; Bongiovanni et al., 2014).

Statistical analysis

The statistical software SPSS (Statistical Package for the Social Sciences) version 19.0 was used for statistical analysis. Analysis of variance (ANOVA) was used for analysing continuous variables. The Chi-square test and the Fisher's exact test were used to study the categorical variables. Pearson's correlation test was performed in order to verify the presence of correlation between values of cleaved caspase-3 and Ki-67. All values were expressed as means ± standard deviation. In all statistical comparisons, $P < 0.05$ was regarded as significant.

Results

Tumours

Benign tumours (n = 18) were classified as simple adenomas (n = 5; 10%), complex adenomas (n = 4; 8%), and benign mixed tumours (n = 9; 18%). Malignant tumours (n = 32) were diagnosed as complex carcinomas (n = 6; 12%), solid carcinomas (n = 6; 12%), tubulopapillary carcinomas (n = 15; 30%) and carcinosarcomas (n = 5; 10%).

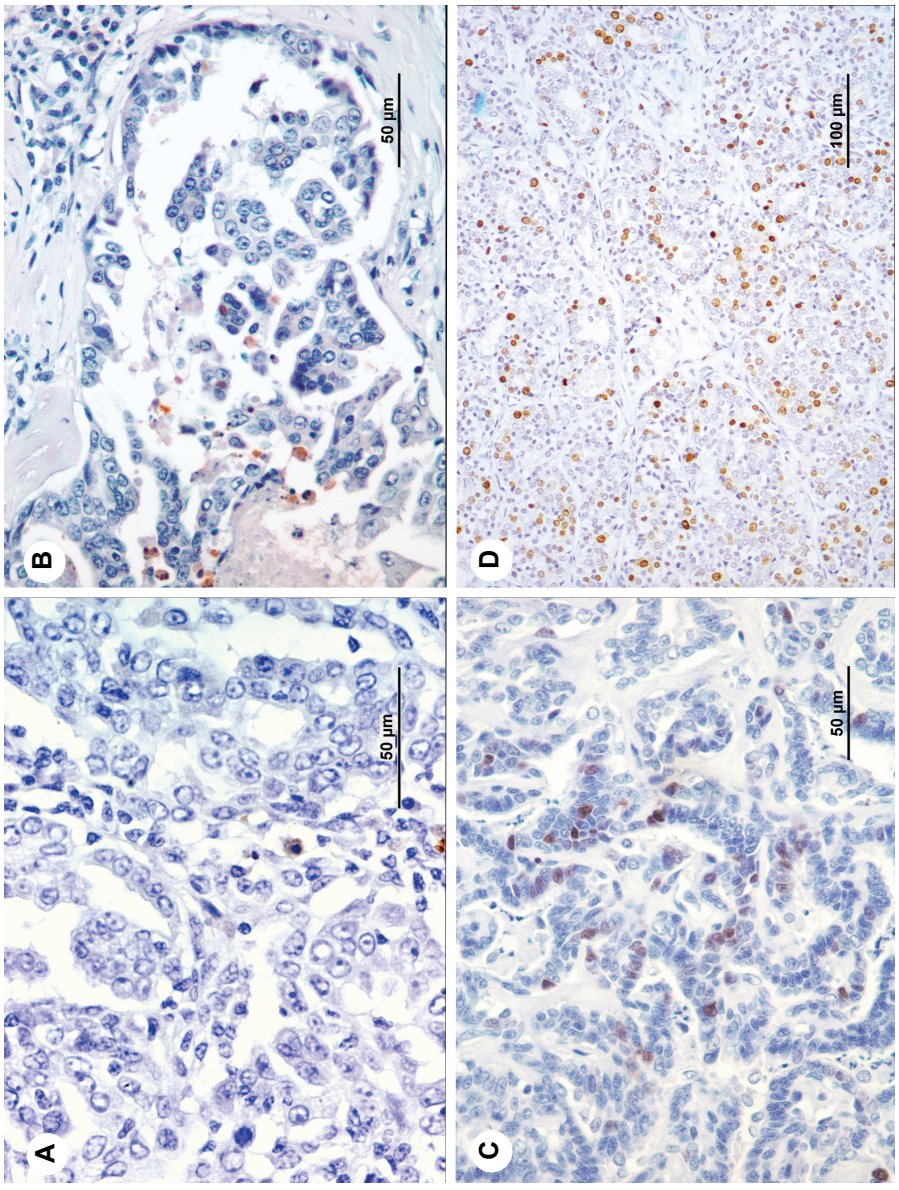

Fig. 1A–D. Canine mammary gland carcinoma. *A.* Low caspase-3 immunoexpression; *B.* High caspase-3 immunoexpression; *C.* Low Ki-67 immunoexpression; *D.* High Ki-67 immunoexpression

Expression of caspase-3 and Ki-67 in canine mammary gland tumours

Immunoreactivity for caspase-3 was observed in the cytoplasm of neoplastic cells in a diffuse pattern. In some cases, nuclear staining was also observed (Fig. 1A,B). Immunostaining for Ki-67 was only observed in the nucleus in a granular labelling pattern associated with the nucleoli. Staining in the nucleoli occurred homogeneously and intensely (Fig. 1C,D).

Differences in the percentage of caspase-3 and Ki-67 positivity between benign and malignant CMGT

A statistically significant difference ($P < 0.001$) in the percentage of caspase-3 positivity was observed in malignant tumours when compared to benign ones (Fig. 2). Malignant tumours showed less apoptotic cells (mean value: 0.647 ± 0.0914%) than benign neoplasms (mean value: 9.478 ± 2.308%). The percentage of Ki-67 observed for malignant and benign CMGT also presented significant differences between the two groups ($P < 0.001$) with higher values recorded for malignant tumours (25.347 ± 2.139%) than for benign neoplasms (7.867 ± 1.0482%) (Fig. 3).

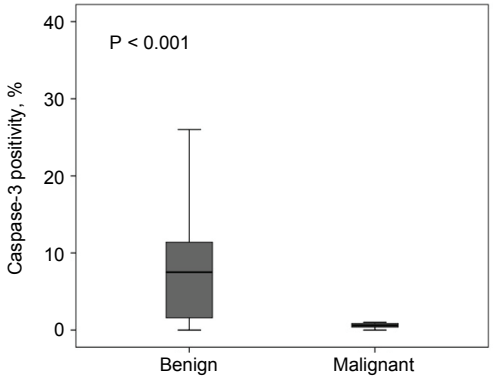

Fig. 2. Differences in caspase-3 positivity (%) between benign and malignant canine mammary gland tumours

Association between the percentage of caspase-3 and Ki-67 positivity and clinicopathological variables in malignant CMGT

The percentage of caspase-3 did not show any association with the clinicopathological variables considered in the study. For the percentage of Ki-67, statistically significant associations were observed with tumour size ($P = 0.025$), histological type ($P = 0.01$), mitotic grade ($P \leq 0.001$), nuclear grade ($P = 0.025$), differentiation grade ($P = 0.004$), histological grade of malignancy ($P = 0.002$), and lymph node metastasis ($P = 0.010$). Detailed information is provided in Table 1.

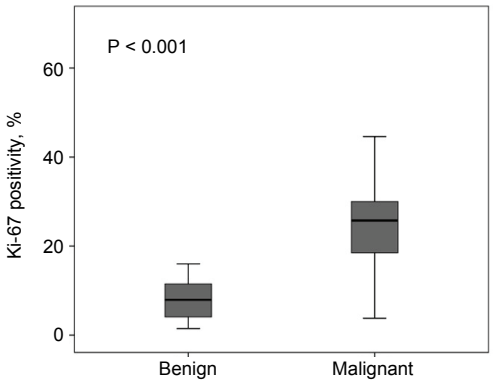

Fig. 3. Differences in Ki-67 positivity (%) between benign and malignant canine mammary gland tumours

Correlation between the percentage of caspase-3 and Ki-67 positivity

In this study, a negative and statistically significant correlation was observed between the percentages of caspase-3 and Ki-67 positivity in the group of malignant tumours ($n = 32$; $r = –0.39$; $P = 0.04$), while no significant correlation was observed in the group of benign tumours.

Discussion

Cell death, particularly apoptosis, is probably one of the most widely-studied subjects in human cancers. Many tumour cells carry mutations in key apoptotic genes such as p53, Bcl family proteins or the genes affecting caspase signalling (Ryoo and Bergmann, 2012).

However, in veterinary medicine only few data have been published about the implication of apoptosis in the diagnosis and/or malignancy of canine tumours and, to the best of our knowledge, there is only a single study that used an antibody to the active form of caspase-3 in these tumours (Bongiovanni et al., 2014).

The present results demonstrated a statistically significant difference in the percentage of caspase-3 positive cells between benign and malignant tumours, with the malignant cases showing less apoptotic cells than benign ones. These findings suggest that caspase-dependent apoptotic pathways are inhibited during tumour progression. The percentage of active caspase-3 was not significantly associated with any clinicopathological feature, and our results are consistent with the findings of a study in canine osteosarcoma (Bongiovanni et al., 2012). In human oncology, several studies have been conducted on this topic, with contradictory results. Some researchers showed that high levels of apoptosis were associated

with the nuclear grade, the degree of differentiation, an increased risk of metastasis and the loss of oestrogen receptors. The relationship between a high apoptotic index and a good prognosis was not substantiated (Lipponen et al., 1994). These inconsistencies in the results may be attributable to the simultaneous existence of several other factors contributing to tumour progression, such as other forms of programmed cell death, rate of proliferation, angiogenesis, and immune response of the host that happen to influence the behaviour of the tumour and its reaction to therapy (Hanahan and Weinberg, 2011). The lack of other studies describing these findings, both in human and veterinary medicine, precludes more adequate comparisons and justifies the need for further investigations in order to clarify this subject.

Cell proliferation is a process controlled by regulatory proteins that guarantee the orderly progression of cells throughout the cell cycle (Madewell, 2001). Anomalies in the activity of these regulatory proteins can lead to increased cell proliferation, poor maintenance of chromosomal integrity, and promotion of tumour growth (Hanahan and Weinberg, 2011). Our results demonstrated that the percentage of Ki-67 positive cells observed for malignant and benign CMGT presented significant differences between the two groups with higher values registered for malignant tumours than for benign neoplasms. These results are in agreement with the findings of previous studies on human breast cancer and on CMGT (Lipponen et al., 1994; Labelle et al., 2004; Nowak et al., 2008; Pathmanathan and Balleine, 2013). These findings highlight the crucial role of Ki-67 in tumour progression, as reported by several authors (Pena et al., 1998; Labelle et al., 2004; Sorenmo et al., 2011). For Ki-67 expression there was a statistically significant association with larger tumour size, histological type, mitotic index, nuclear grade, histological grade of malignancy, and the presence of lymph node metastasis. Similar results have already been described by other researchers for human and canine mammary tumours (Labelle et al., 2004; De Matos et al., 2006; Richardsen et al., 2012; Santos et al., 2013).

Our data also demonstrated a statistically significant negative correlation between active caspase-3 and Ki-67 expression in the malignant tumour group. The rate of tumour growth is determined by cell death and proliferation. An imbalance between tumour cell proliferation and death is critical for the growth, progression and regression of the tumour (Hanahan and Weinberg, 2011). Therefore, qualitative and quantitative changes in the death of tumour cells with proliferative changes are essential and determinant in the pathogenesis of malignant disease and its responsiveness to therapy (Okada and Mak, 2004).

Our study suggests a loss of balance between cell death and cell division in CMGT. In these tumours, the pathways implicated in cell death and proliferation were altered and tumour cells that should be detected as foreign were not appropriately signalled.

Table 1

Relationship between caspase-3 and Ki-67 expression (%) and clinicopathological parameters in malignant canine mammary tumours

Clinicopathological parameters	Ki-67			Caspase-3		
	Low n	High n	P	Low n	High n	**P**
Tumour size						
T1 < 3 cm	6	4	0.025	7	3	NS
T2 ≥ 3 cm and < 5 cm	1	8		4	5	
T3 ≥ 5 cm	2	11		8	5	
Skin ulceration						
Absent	16	9	NS	14	11	NS
Present	0	7		5	2	
Histological type						
Tubulopapillary c.	4	11	0.010	11	4	NS
Solid c.	0	6		2	4	
Complex c.	5	1		2	4	
Carcinosarcoma	0	5		1	2	
Tumour necrosis						
Absent	8	13	NS	12	9	NS
Present	1	10		7	4	
Mitotic index						
I	8	1	< 0.001	5	4	NS
II	0	9		6	3	
III	1	13		8	6	
Nuclear grade						
I	0	0	0.025	0	0	NS
II	6	5		7	4	
III	3	18		12	9	
Differentiation grade						
I	4	1	0.004	3	2	NS
II	4	7		7	4	
III	1	15		9	7	
Histological grade of malignancy						
I	6	2	0.002	5	3	NS
II	2	6		4	4	
III	1	15		10	6	
Lymph node involvement						
N_0	9	12	0.010	11	10	NS
N_+	0	11		8	3	

n: number of samples; P: statistical significance; NS: not significant

Cell survival, proliferation, migration, metabolism, angiogenesis and apoptosis are mediated by a range of pivotal signalling pathways that have a regulatory function, such as PI3K/AKT/mTOR and Ras-Raf/MEK/ERK signalling (Fruman and Rommel, 2014). Abnormal activation of these pathways, caused by PI3K mutation, KRAS mutation, PTEN loss, AKT1 mutation and other mechanisms, is possibly the most commonly activated deregulated proliferation and apoptotic signalling pathway in human breast cancer (Liu et al., 2009; Hernandez-Aya and Gonzalez-Angulo, 2011). Many studies showed that more than 70% of breast tumours have molecular alterations in at least one component of these pathways (Hernandez-Aya and Gonzalez-Angulo, 2011), and our results suggest that in CMGT similar pathways could be involved. Although the deregulated cell proliferation and the suppression of apoptosis that are needed to support neoplastic progression can be activated simultaneously by the same stimuli, they might sometimes be regulated by different and independent signal and mutation types (Ryoo and Bergmann, 2012), which claims for additional research on this topic.

Understanding the network of apoptosis and proliferation in CMGT is very important as it not only gives insights into the pathogenesis of the disease, but may also leave clues on how the disease can be treated.

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