FULL PAPER *Surgery*

Relationship between NF-κB Expression and Malignancy of Canine Mammary Gland Tumor Tissues

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ABSTRACT. In this study, the nuclear expression of nuclear factor kappa B (NF-κB) in 48 tissues specimens from 25 canine spontaneous mammary gland tumor (MGT) patients was assessed by immunohistochemistry to compare their levels with clinical features, histological types, prognostic outcomes and proliferative activities, including the mitotic index (MI) and cylcinD1 expression. Twelve of eighteen (66.7%) malignant tumor tissues showed greater than 10% nuclear staining, while benign tumor and hyperplastic tissues showed less than 10% nuclear staining. Higher nuclear expression of NF-κB was positively correlated with larger tumor size, lymph node metastasis and higher MI; however, no correlation was observed with distant metastasis and cyclin D1 expression. Higher NF-κB nuclear expression correlated with shorter patient survival. These findings suggest that NF-κB is a useful prognostic factor for canine MGT patients.

KEY WORDS: canine MGT, immunohistochemistry, malignancy, NF-κB, prognosis.

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Mammary gland tumor (MGT) is the most common type of tumor in intact female dogs [11]. The histological type of MGT tissues is strongly related to prognosis, with negative prognosis being associated with poorly differentiated tumors. Among the related factors, distant metastasis is the most significant, and dogs with distant metastasis have a worse prognosis than those with only regional lymph node involvement [15, 17].

Progression of breast cancer from a benign to a malignant phenotype is accompanied by overexpression of several growth factors, cytokines, and chemokines in cancer cells [22]. Some of these substances can induce the expression of prometastatic genes through nuclear factor kappa B (NF-κB) [22]. Elevated DNA binding activity of NF-κB was observed in breast cancer cell lines with invasive and metastatic growth properties [22]. The NF-κB complex is composed of a family of inducible transcription factors found in almost all cells [3, 4, 13], and this complex is generally recognized as an essential cell mediator acting "at the crossroads of life and death" [16]. The mammalian NF-κB family consists of 5 members: p50 (NF-κB1), p52 (NF-κB2), p65 (Rel A), c-Rel, and Rel B [23]. These Rel family members exist as homo- or heterodimers, and the most abundant form of intracellular NF-κB is generally the p50/p65 heterodimer [23]. In resting cells, NF-κB is cytoplasmically sequestered as a latent complex bound to one or more members of the IκB protein family (I-κBα, I-κBβ, I-κBε, I-κBγ, Bcl-3 and the precursor Rel proteins p100 and p105). Diverse cell stimuli induce phosphorylation (via activation of the I-κB kinase complex) and subsequent proteasomal degradation of I-κB inhibitory proteins, leading to the activation of NF-κB for nuclear translocation and DNA binding [23]. Nuclear traslocated NF-κB is reported to activate the transcription of many factors including cyclin D1, which is a key regulator of G1 checkpoint control [7, 23].

Information about the significance of NF-κB nuclear expression in the canine MGT is limited. Vinothini *et al.* reported higher protein expression of NF-κB in an advanced stage but did not mention the nuclear expression and prognosis [29]. In this study, 48 tissue specimens from 25 canine spontaneous MGT patients were evaluated for the nuclear expression percentage of NF-κB to compare to their histopathological types, clinical malignancy, and proliferative activities [mitotic index (MI) and cyclin D1 expression]. We also evaluated the relationship between the NF-κB expression and their prognosis.

MATERIALS AND METHODS

Tissue samples: Forty-eight canine spontaneous MGT tissues were obtained from 25 dogs who underwent surgical resection at the Veterinary Medical Center (VMC) of The University of Tokyo between June 2006 and November 2008. None of the patients received any chemotherapeutics

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or anti-inflammatories before surgery, which may influence NF-κB activity. Four normal mammary tissues were obtained from healthy beagles with no tumors that were euthanized for other experiments. Clinical information, including patient breed, age, body weight, history of spaying before the first estrus, tumor size, regional lymph node involvement, distant metastasis and prognostic outcome, were obtained from medical records or a telephone interview with the owner. The lymph node involvement was evaluated by palpation prior to surgery and histologically confirmed after surgery. Lung metastasis was evaluated on thoracic radiography. The mean follow-up time was 611 days (range, 1–1,190 days).

The surgical specimens were fixed in 10% buffered formalin, processed, and embedded in paraffin according to the standard protocol. Histological diagnosis was made by diplomates of the Japanese College of Veterinary Pathologists in the Laboratory of Veterinary Pathology, the University of Tokyo. The TNM classification stage was used for the classification of MGT staging [18]. Survival time was defined as the time from surgery to death.

Immunohistochemistry: The primary antibodies used for immunohistochemistry (IHC) were the monoclonal antibody against NF-κB (p65) (BD Transduction Laboratories, Lexington, KY, U.S.A.) and the polyclonal antibody against cyclin D1 (Clone M20, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) [21]. Both antibodies were used at 1/50 dilution.

Formalin-fixed, paraffin-embedded, 5-μm-thick sections were deparaffinized in xylene and rehydrated through graded ethanol followed by distilled water. Sections were then subjected to heat antigen retrieval by autoclaving in citrate buffer (pH=6.0) at 120°C for 10 min. Endogenous peroxidase activity was inhibited using 3% H_2O_2, and the nonspecific antibody binding was reduced by incubating the sections with 10% normal goat serum (Dako, Glostrup, Denmark) in Tris-buffered saline and Tween 20 (TBS-T) for 1 hr at room temperature. The primary antibody was diluted in the same solution. The mixture was incubated at 4°C overnight, and treated with the secondary antibody for 30 min and then treated with EnVision (Dako). Visualization was performed using DAB (Dako). Finally, tissues were counterstained with hematoxylin and dehydrated through graded alcohol and xylene. The positive controls for NF-κB and cyclin D1 staining were normal canine lymph node and one known cyclin D1-positive canine MGT tissue, respectively. Primary antibody was substituted with TBS in the negative controls.

Only nuclear-stained cells were considered to be positive and the percentage of nuclear staining was counted in 5 random fields in each slide using a light microscope at 400× magnification. Expression of NF-κB was assessed according to the following scoring: Score 1 when the immunoreactivity was <10%, Score 2 when the immunoreactivity was between 10 and 20%, and Score 3 when >20% of cells showed positive immunoreactivity.

MI: MI was counted for a series of 5-μm-thick sections with hematoxylin-eosin stain under light microscopy (400× magnification). MI was calculated by the following formula: MI=number of cells containing visible chromosomes × 100/ total number of cells in the field of view. Mitotic figures were counted in 5 random fields in each slide, and the mean of these 5 fields was used for calculating MI. MI was assessed according to the following scoring: Score 1, the mitotic figures occupied <0.1% of the chosen fields; Score 2, the mitotic figures occupied 0.1–0.5% of the chosen fields; Score 3, the mitotic figures occupied >0.5% of the chosen fields.

Statistical analysis: Comparisons of NF-κB nuclear expression, MI and cyclin D1 nuclear staining positive-rates with histopathological types were made by the Kruskal-Wallis test and the Chi-square test. The Pearson correlation coefficient was used to investigate the correlation between NF-κB nuclear expression, MI percentage, cyclin D1 nuclear expression, tumor size and clinical stage. Survival curves in the groups according to NF-κB expression score were obtained using the Kaplan-Meier method and were compared using the log-rank test to assess statistical significance. A probability of less than 5% (P<0.05) was considered statistically significant.

RESULTS

Clinical and histopathological features of canine MGT patients: A total of 25 dogs with MGT, consisting of 21 intact and 4 spayed females (spayed between 6 and 12 years of age), were used in this study. These dogs underwent at least 1 surgical operation for mammary tumor removal. The surgeries were unilateral or partial mastectomies performed with curative intent. The mean age of the patients at the first surgery was 10.7 years (range, 5.8–15.2 years). The breed distribution was as follows: 5 miniature Dachshunds, 4 Yorkshire terriers, 3 Malteses, 2 Italian greyhounds, 2 mixed, 2 Shetland sheepdogs, 2 Siberian huskies, 1 Cavalier King Charles spaniel, 1 miniature schnauzer, 1 shih-tzu, 1 toy poodle, and 1 Welsh corgi. The mean body weight was 8.2 kg, (range, 1.5–21 kg). The histopathological diagnoses of the 48 tissues are shown in Table 1. There were 18 adenocarcinomas, 10 adenomas, 9 benign mixed tumors and 11 hyperplasias. Table 2 shows the TNM classifications of the patients with MGT. Classification of tumor size was based on the most malignant tissue mass of each patient. Most of the patients had masses <3 cm in size. Regional lymph node involvement was histologically observed in 4 of 25 dogs, and pulmonary metastasis was found in 2 patients.

Immunohistochemistry: Almost all the tissue samples showed cytoplasmic staining; however, only nuclear localization of NF-κB was considered positive (Fig. 1). All stromal cells were excluded in counting stained cells. Table 3 shows the nuclear expression of NF-κB in canine MGT tissues. All except 4 (22.2%) of the adenocarcinoma tissues showed higher NF-κB expression levels; these 4 tissues had <10% of NF-κB expression (Score 1). On the other hand, all the adenomas, benign mixed tumors and hyperplasias revealed NF-κB nuclear expression in <10% of cells. Healthy mammary gland tissues showed no NF-κB expression. Six

Table 1. Histopathological diagnosis of MGT tissues

Histopathological diagnosis		Number of samples	Total
Adenocarcinoma	Adenocarcinoma	12	18
	Complex carcinoma	3	
	Schirrhous carcinoma	2	
	Solid carcinoma	1	
Adenoma	Adenoma	7	10
	Complex adenoma	3	
Benign mixed tumor		9	9
Hyperplasia		11	11
	Total		48

Table 2. TNM classification of the 25 the patients with MGT

	Characteristic	Number of patients
T	T1 <3	14
	T2 3–5	7
	T3 >5	4
N	Negative (N0)	21
	Positive (N1)	4
M	Negative (M0)	23
	Positive (M1)	2

adenocarcinoma tissues (33.3%) were categorized as Score 2, and the remaining 8 adenocarcinoma tissues (27.8%) were categorized as Score 3. A significant difference ($P<0.0001$) was detected between adenocarcinomas and all other histopathological types, whereas insignificant differences were noted between the normal tissues and the benign mixed tumors, adenomas, and hyperplasias (Table 3). There was no significant difference in NF-κB nuclear expression in benign tissues when compared between malignant and benign MGT patients.

The expression of cyclin D1 varied throughout the tissues of the same histopathological type, and there was no significant difference among them.

MI: Table 4 summarizes the MI scores of each histopathological type. All adenomas (100%), 7 benign mixed tumors (77.8%) and 8 hyperplasias (72.7%) had an MI of <0.1% (Score 1); 7 adenocarcinomas (38.9%) had an MI of 0.1–0.5% (Score 2); and 5 adenocarcinomas (27.8%) had an MI of >0.5% (Score 3). One patient (11.1%) with a benign mixed tumor had an MI >0.5%. No mitotic figures were detected in normal mammary gland tissues. A significant difference in MI values was detected between adenocarcinomas and adenomas ($P=0.044$).

Correlation of NF-κB expression with clinical features, MI and cyclin D1: According to the TNM classification,

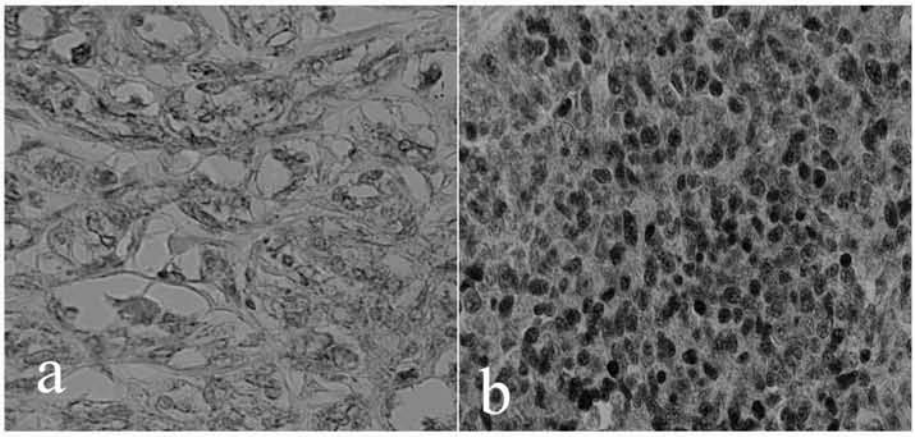

Fig. 1. a) NF-κB expression in a benign mixed tumor tissue showing cytoplasmic staining with lower nuclear staining (IHC: 400× magnification). b) NF-κB expression in an adenocarcinoma tissue showing higher nuclear staining, which was considered to be positive NF-κB staining (IHC: 400× magnifications).

Table 3. NF-κB nuclear expression scores according to each histopathological type

	Number (%)				Mean (± SD)	
	Score 1	Score 2	Score 3	Total		
Adenocarcinoma	4 (22.2)	6 (33.3)	8 (44.5)	18 (100)	18 (± 14.2)	*
Adenoma	10 (100)	0 (0)	0 (0)	10 (100)	1.7 (± 1.5)	
Benign mixed tumor	9 (100)	0 (0)	0 (0)	9 (100)	1.8 (± 2.9)	
Hyperplasia	11 (100)	0 (0)	0 (0)	11 (100)	3.8 (± 3)	
Normal mammary gland	4 (100)	0 (0)	0 (0)	4 (100)	0	

*There was a significant difference in NF-κB nuclear expression between adenocarcinoma and all other histologic types ($P<0.0001$).

Table 4. Mitotic index scores according to each histopathological type

	Number (%)				Mean (± SD)	
	Score 1	Score 2	Score 3	Total		
Adenocarcinoma	6 (33.3)	7 (38.9)	5 (27.8)	18 (100)	0.5 (± 0.5)	*1
Adenoma	10 (100)	0 (0)	0 (0)	10 (100)	0.02 (± 0.03)	
Benign mixed tumor	7 (77.8)	1 (11.1)	1 (11.1)	9 (100)	0.1 (± 0.3)	
Hyperplasia	8 (72.7)	3 (27.3)	0 (0)	11 (100)	0.1 (± 0.001)	
Normal mammary gland	4 (100)	0 (0)	0 (0)	4 (100)	0	

*There was a significant difference in MI between adenocarcinoma and adenoma tissues (P=0.044).

Table 5. Correlation of NF-κB nuclear expression with TNM classification and MI

Score of NF-κB*	Tumor size			LN metastasis		Distant metastasis		Mitotic index*		
	T1	T2	T3	N0	N1	M0	M1	S1	S2	S3
S1 (%)	11 (73)	2 (33)	0 (0)	14 (67)	0 (0)	14 (61)	0 (0)	27 (87)	4 (36)	3 (50)
S2 (%)	1 (7)	2 (33)	0 (0)	3 (14)	0 (0)	1(4)	0 (0)	2 (6)	3 (27)	1(17)
S3 (%)	3 (20)	2 (33)	3 (100)	4 (19)	4 (100)	8(35)	2 (100)	2 (6)	4 (36)	2 (33)
P		0.023		0.017		0.361			0.001	
r		0.543		0.472		0.191			0.459	

The product-moment correlation test was used in the analysis. *S1, S2 and S3: Score1, 2 and 3, respectively.

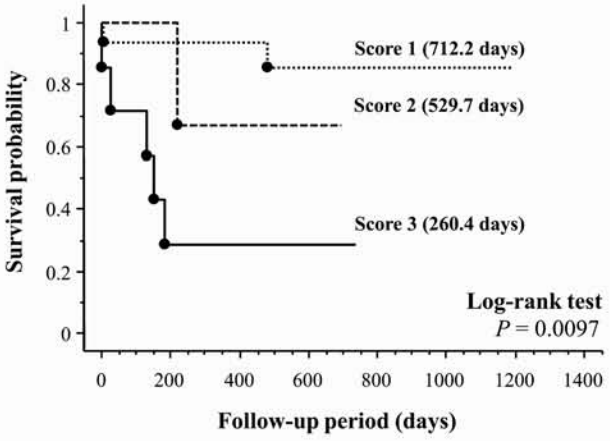

Fig. 2. Kaplan-Meier survival curve of canine MGT patients grouped according to the level of NF-κB nuclear expression scores. A significant difference was detected among the 3 groups (P=0.0097).

the NF-κB nuclear expression values of the most malignant tissue of each patient were used for the analysis. Eleven of 15 dogs (73%) with tumors <3 cm in size (T1) showed NF-κB nuclear expression<10% in their tumor masses, while all 3 dogs with tumors >5 cm in size (T3) showed NF-κB nuclear expression >20%. Fourteen of 21 dogs (67%) with no lymph node involvement (N0) showed NF-κB expression <10%, which was in contrast to all 4 dogs with lymph node involvement (N1), who showed NF-κB nuclear expression >20%. Similarly, 14 of 23 dogs without distant metastasis showed low NF-κB expression, while 2 dogs with distant metastasis showed high expression (Table 5). A positive correlation between NF-κB nuclear expression and tumor size (r=0.453, P=0.023) and NF-κB nuclear expression and lymph node metastasis (r=0.472, P=0.017) was observed; however, no correlation was detected between NF-κB activation and distant metastasis (r=0.191, P=0.361).

The relationship between NF-κB expression and patient survival is shown in Fig. 2. The median survival time was 798, 674 and 154 days in dogs with NF-κB Scores of 1, 2 and 3, respectively. One dog with a Score 1 adenoma died of causes other than MGT 5 days after the surgery. Another dog with a Score 3 adenocarcinoma died during the operation, and autopsy revealed the presence of lung metastasis. The two-year survival rates of patients with Scores 1, 2 and 3 were 86.7, 66.7 and 28.6%, respectively. By using the log-rank test, a significant difference was detected among these 3 groups (P=0.0097).

The correlation between nuclear expression of NF-κB and MI is shown in Table 5. A positive correlation was revealed between NF-κB nuclear expression and MI (r=0.459, P=0.001). No correlation was found between NF-κB and cyclin D1 nuclear expression (r=0.234, P=0.175) or between MI and cyclin D1 (r=0.142, P=0.312).

DISCUSSION

NF-κB is an important cellular regulator of growth and apoptosis. The predominant mechanism of regulation of this transcriptional factor activity is through the canonical pathway; IKK-β is activated by the stimuli of inflammatory cytokine signaling, infectious agents and DNA damage, and the activated IKK-β phosphorylates the I-κB protein. Then phosphorylated I-κB induces the nuclear translocation of NF-κB (p65-50 heterodimers) [25]. NF- κB has been reported to be associated with multiple aspects of oncogenesis, including control of apoptosis, the cell cycle, differentiation and migration [5, 27]. Constitutive NF-κB activity has been

found in a wide variety of tumors, suggesting a strong correlation between NF-κB expression and tumorigenesis [6, 8]. In the present study, we focused on the nuclear p65 expression as the nuclear translocated NF-κB and investigated its expression in 48 canine MGT tissue samples. However other molecules related to NF-κB need further study.

Adenocarcinoma tissues showed the highest nuclear expression of NF-κB. High nuclear expression of NF-κB in these tissues was correlated with a high MI. On the other hand, most of the benign tissues showed lower NF-κB nuclear localization (<10%) and lower MI (<0.1%). These findings permit us to conclude a positive correlation between MGT malignancy and NF-κB nuclear expression, which supports the results of previous studies in canine MGTs [29] and in human breast cancers [10, 24]. In the present study, 9 of 25 dogs had multiple masses in their mammary glands.

The correlation between NF-κB nuclear expression and patient clinical features was assessed. Four of the 25 dogs had lymph node involvement; in 3 of these 4 dogs, lymph node involvement was histopathologically confirmed. A positive correlation was found between NF-κB nuclear expression and tumor size and between NF-κB nuclear expression and lymph node metastasis, but no correlation was found between NF-κB nuclear expression and distant metastasis. Distant metastasis was detected only in 2 dogs, both of which died early in the disease course due to metastasis. The number of patients bearing metastasis in this study may not be sufficient for detection of any correlation between NF-κB nuclear expression and distant metastasis.

NF-κB nuclear expression was correlated with shorter survival, with a statistically significant difference between the patients with the 3 scores of NF-κB expressions according to the log-rank test. Therefore, NF-κB is a useful prognostic factor in canine MGT. Higher expression of NF-κB may be associated with worse patient outcome. Of the 5 dogs who died due to MGT, 4 presented with Score 3 nuclear expression of NF-κB, and one presented with Score 1 (slightly <10%). These findings agree with the results of Liu C. *et al.*, who used 130 human breast cancer tissue samples and found that upregulation of NF-κ B expression indicated poor prognosis of breast cancer with lymph node metastasis and that it was also related to an enhanced risk of recurrence and metastasis [19].

In the present study, cyclin D1 nuclear staining did not correlate with either NF-κB nuclear localization or MI. In addition, no significant difference in cyclin D1-nuclear staining was recorded among tissues with different histopathological types. The role of cyclin D1 in breast cancer prognosis remains unclear. Some studies have shown a correlation between cyclin D1 expression and poor or good prognosis, while some showed no correlation between them [1, 2, 14, 26, 28]. Experimental data implied that cyclin D1 was needed to drive proliferation in estrogen receptor (ER)-positive breast cancer cells, while in ER-negative breast cancer cells, cell proliferation proceeds through other cyclin D1-independent mechanisms [20]. Further research is needed to clarify the role of cyclin D1 in canine MGT. In this study, no correlation was found between MI and cyclin D1 expression. This finding agrees with the results of Nurija B. *et al.*, who did not find a correlation between cyclin D1 expression and MI in human breast cancer specimens [9]. In some other tumors, like lung cancers and colorectal cancers, the expression of cyclin D1 was correlated with worse outcome and had a positive correlation with proliferative markers. This may indicate that cyclin D1 activities might be not only diverse but also tissue specific [12].

In conclusion, NF-κB activation was correlated with high proliferation potential, larger tumor size and lymph node metastasis in canine MGTs. Furthermore, NF-κB nuclear expression was revealed to correlate with shorter patient survival. NF-κB is a useful prognostic factor in canine MGTs. However, the sample size was limited, and histopathological grading was not adopted in this study. Further studies with a larger patient population with histopathologically confirmed lymph node involvement and lung metastasis are needed to clarify the relationship between NF-κB activation and metastasis.

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