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Evaluation of FOXP3 Expression in
Canine Mammary Gland Tumours

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Evaluation of FOXP3 Expression in Canine Mammary Gland Tumours

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Abstract

Forkhead box P3 (FOXP3) is a member of the forkhead/winged-helix family of transcription regulators involved in regulating immune system development and function. This gene plays a crucial role in the generation of $CD4^{+}CD25^{+}$ regulatory T cells (Tregs). In addition, Tregs have an important role in cancer, as they enable tumors to elude the host

antitumor immune response. In a recent human research suggested that FOXP3 expression is a novel, independent molecular marker of human breast carcinoma, with a significant impact on important outcome measures for breast carcinoma.

The purpose of this study is to evaluate the expression of FOXP3 in canine mammary gland tumours and to investigate its prognostic possibility in canine mammary gland tumours comparing to other known prognostic markers.

In this study, the immunohistochemical expression of FOXP3 in canine mammary gland tumors and its relationship with some other known clinicopathologic parameters, such as hormone receptors (ER and PR), human epidermal growth factor receptor 2 (HER2), and survival, were evaluated to investigate the possible value of FOXP3 as a prognostic factor in canine mammary gland tumors. Among the 62 dogs, forty dogs had benign mammary gland tumors and twenty two dogs had malignant mammary gland tumors. The patterns of FOXP3 expression was heterogeneous and ranged from mostly cytoplasmic to both cytoplasmic and nuclear. Positive FOXP3 expression was observed in 5 % of benign mammary gland tumours, 54.5 % of malignant mammary gland tumours and 22.6 % of total mammary gland tumours. ER, PR and HER2 expression was observed in 87.5 %, 92.5 % and 5 % of benign mammary gland tumours, 18.2 %, 18.2 % and 18.2 % of malignant mammary gland tumours. Relationship between the FOXP3 expression and several clinicopathologic

parameter, positive staining for FOXP3 was associated with the tumour size (P=0.025), presence of inflammation (P=0.043) and histological type (P<0.001) but not with age, breed, lymph node metastasis and distant metastasis.

Associations between the FOXP3 expression and other molecular markers which already known prognostic markers in canine mammary gland tumours, negative staining for FOXP3 was associated with positive ERα and PR expression (P<0.001) but not with HER2 expression.

The expression of FOXP3 in canine mammary gland tumours was significantly associated with the decreased disease-free survival time (P=0.029).

The FOXP3 expression was not an independent prognostic factor in the multivariate analysis, though. The negative expression of FOXP3 in the canine mammary gland tumours was found to be had high prediction rate for benign mammary gland tumours and a longer disease free survival (DFS) time in canine mammary gland tumours.

Keywords: Dog, FOXP3, Immunohistochemistry, Mammary gland tumour, Prognostic factor

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CONTENTS

I. Introduction

Mammary gland tumor is the most common neoplasm in female dogs. It represents a significantly heterogeneous group in terms of morphology and biological behavior (Nerurkar et al. 1989; Rutteman et al. 2001). There are some recognized and well-accepted prognostic factors of malignant mammary tumors in dogs, such as the tumor size, lymph node status, distant metastasis, histologic type, histologic malignancy grade, and degree of nuclear differentiation (Misdorp and Hart 1976; Rutteman, Withrow et al. 2001). Moreover, in recent literature, an increasing number of investigations of suitable prognostic markers of canine mammary tumors was found (Zaidan Dagli 2008), which included proliferation markers (De Matos et al. 2006), hormone receptors (de las Mulas et al. 2005), p53 and human epidermal growth factor receptor 2 (HER2) (Martin de las Mulas et al. 2003; Lee et al. 2004), and adhesion molecules (De Matos et al. 2007; Gama et al. 2008), among others.

Estrogen and progesterones play major roles in normal development of the mammary glands. But these hormones have also play an important role in the pathogenesis of canine mammary gland tumours (Schneider et al. 1969). In dogs, both benign and malignant mammary gland tumours may express ERα (Donnay et al. 1995) and PR (de las Mulas,

Millán et al. 2005). However, ERα expression was found to be higher in benign tumours than in malignant tumours (Nieto et al. 2000). Lack of PR was associated with a high histologic grade, tumour invasion, lymph node involvement, or metastasis (de las Mulas, Millán et al. 2005). These hormone receptors have proven their usefulness in characterizing subgroups with different prognosis among canine mammary gland tumours (de las Mulas, Millán et al. 2005).

HER2 oncogene was found to contribute to tumourigenesis of human breast cancers (Slamon et al. 1987). Overexpression of HER2 tends to have shorter DFS, shorter survival time and generally a poor prognosis in human breast cancer (Almasri and Al Hamad 2005). However in veterinary field, one research found that dogs with malignant mammary gland tumours with HER2 overexpression generally had a higher survival rate for 2 years after surgical removal of the tumours, compared with the survival rate for dogs with mammary gland tumours with typical HER2 expression (Hsu et al. 2009). Immunohistochemical overexpression of HER2 in canine mammary gland tumor has variable result. 19.4% (Rungsipipat et al. 1999) and 74% (Ahern et al. 1996). It indicated that additional investigations of HER2 overexpression in canine mammary gland tumours.

Forkhead box P3 (FOXP3) is a member of the forkhead/winged-helix family of transcription regulators involved in regulating immune system development and function (Coffer and Burgering 2004). This gene plays a crucial role in the generation of $CD4^{+}CD25^{+}$ regulatory T cells (Tregs). The loss of FOXP3 function leads to the lack of Tregs, which results in lethal auto-aggressive lympho-proliferation, whereas over-expression of FOXP3 results in severe immunodeficiency (Hori et al. 2003). In addition, Tregs have an important role in cancer, as they enable tumors to elude the host antitumor immune response. In human research, higher numbers of Tregs in the peripheral blood of patients with breast (Liyanage et al. 2002), liver (Ormandy et al. 2005), gastric, and esophageal cancer (Ichihara et al. 2003) than in healthy controls have been reported. Furthermore, increased numbers of tumor-infiltrating Tregs have been demonstrated in ovarian (Curiel et al. 2004), hepatocellular (Ormandy, Hillemann et al. 2005), gastric, and esophageal cancer (Ichihara, Kono et al. 2003). Moreover, a recent study suggested that FOXP3 expression is a novel, independent molecular marker of human breast carcinoma, with a significant impact on important outcome measures for breast carcinoma (Merlo et al. 2009).

In this study, the immunohistochemical expression of FOXP3 in canine mammary gland tumours was evaluated. And also its relationship with some other known

clinicopathologic parameters and survival were evaluated. Finally, we evaluate the relationship between FOXP3 expression and some proven prognostic factors in canine mammary gland tumours, such as hormone receptors (ER and PR) and HER2 to investigate the possible value of FOXP3 as a prognostic factor in canine mammary gland tumours.

II. Materials and Methods

1. Animals

A total of 62 female dogs with mammary gland tumors (40 of which had benign tumors and 22 malignant tumors) were identified for use in this study. The dogs had been examined at Seoul National University Veterinary Medical Teaching Hospital between January 2006 and December 2010. They were appropriate for inclusion when a diagnosis of a mammary gland tumor had been confirmed via a histologic examination (Misdorp et al. 1999). Samples were obtained via surgical excision (simple, regional, unilateral, and total mastectomy) on each dog that was admitted to a veterinary clinic for clinical evaluation and treatment of a mammary gland tumor. The type of surgical resection was determined based on the size, location, and number of tumors.

2. Data collection

Clinical information was collected from medical records. The information collected included the breed, sex, age, body weight, ovariohysterectomy (OHE) status, tumor size, inflammation, regional lymph node status, presence of distant metastasis, clinical stages, type of surgery, and disease free survival (DFS) time. The clinical stages were determined

using the classification of the World Health Organization (WHO) (Misdorp, Else et al. 1999). The tumor size was classified as T1 (< 3 cm), T2 (3-5 cm), or T3 (> 5 cm). N indicated the condition of the regional lymph nodes, and M, the presence of distant metastasis.

3. Histological examination

The samples were fixed in 10% neutral buffered formalin and then embedded in paraffin wax. A 4 μm-thick section was cut from each tumor sample block and placed on a silane-coated slide glass (MUTO, Tokyo, Japan). The tumor slides were stained with haematoxylin and eosin (HE). Thereafter, each slide was evaluated microscopically and classified using the diagnostic criteria suggested by the World Health Organization classification of tumors in domestic animals (Misdorp, Else et al. 1999).

4. Immunohistochemistry

The tumor sections were deparaffinised in xylene and rehydrated in graded alcohol. Antigen retrieval was carried out with a 2100-retriever pressure cooker (PickCell Laboratories, Amsterdam, Netherlands) in a 10 mM citrate buffer, at a pH of 6.0 for ERα, PR, and HER2, and with a 1 mM ethylene diamine tetraacetic acid (EDTA) buffer for FOXP3. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS

for 15 min and 10% normal goat serum in 0.01 M PBS for 30 min. Then they were incubated overnight with primary antibodies against ERα, PR, HER2, and FOXP3. Table 1 summarizes the antibodies used and the staining protocol. After the PBS washing, each slide was incubated with a biotinylated secondary antibody and streptavidin-conjugated peroxidase. Subsequently, 3, 3-diaminobenzidine tetra-hydrochloride (DAB) was used for color development. The slides were then counterstained with haematoxylin and dehydrated. Immuno horseradish peroxidase-DAB anti-mouse IgG (H+L) kit (ImmunoBioscience Corp., Mukilteo, Washington, USA) was used for each step, from the quenching to the counterstaining. Finally, the sections were mounted with Canada balsam (Kanto, Tokyo, Japan) for microscopic evaluation. Normal canine mammary gland tissues were used for positive control of ERα and PR. And normal lymph node was used for positive control of FOXP3 based on previous study in canine tumor patient (Biller et al. 2007). A negative control test was carried out using PBS instead of a primary antibody.

Table 1. Primary antibodies and immunostaining protocols

Marker	Origin	Clone	Host	Dilution
ERα	Dakocytomation	M7047	Mouse	1:35
PR	Beckman Coulter	IM1546	Mouse	1:250
HER2	Novocastra	NCL-CB11-L	Mouse	1:40
FOXP3	eBioscience	FJK-16s	Mouse	1:250

5. Evaluation of the immunohistochemical data

The staining proportion and staining intensity for ERα and PR expression were evaluated using the previously established immunoperoxidase scoring system (Allred et al. 1998). The percentage of tumor cells with positive staining (proportion score or PS) was graded from 0 to 3 via estimation (0 = less than 5% staining; 1 = 5-19%; 2 = 20-60%; and 3 = more than 60% of the tumor nuclei showed positive staining). The staining intensity (intensity score or IS) estimated the average staining intensity of the positive tumor cells and was scored from 0 to 3 (0 = negative; 1 = slight staining; 2 = moderate staining; and 3 = strong staining). The PS and IS were added to obtain the total score (TS) (range: 0-6). ERα and PR were considered positive when PS was ≥ 1 and TS ≥ 2.

The HER2 expression was evaluated using the HercepTest scoring system (Hsu et al. 2002). The staining intensity was scored from 0 to 3+ via estimation (0 = no staining or membrane staining in < 10% of the tumor cells; 1+ = faint / largely incomplete membrane staining in > 10% of the tumor cells; 2+ = weak to moderately complete membrane staining in > 10% of the tumor cells; and 3+ = strong complete membrane staining in > 10% of the tumor cells), with 2+ and 3+ cases considered overexpressed.

The FOXP3 expression was evaluated using the previous human FOXP3 scoring system (Merlo, Casalini et al. 2009). The percentage of tumor cells with positive staining of at least 25% was considered positive for FOXP3 expression (Table 2). Two blinded investigators evaluated the marker expression. Each sample slides were observed in 10 ~ 15 random fields. Counts of the positive cells were recorded in a total of 1000 tumor cells.

Table 2. Evaluation of immunohistochemical FOXP3 expression.

Evaluation	Interpretation	Staining status
Negative	Negative	Negative staining or staining < 25 % of neoplastic cells
Positive	Weak	Slightly staining of at least > 25 % of neoplastic cells
	Strong	Strong staining of at least > 25 % of neoplastic cells

6. Follow-up

All the dogs were followed up post-surgically by telephone with the clients and referral hospital. Their DFS was defined as the time from their surgery to the date of their tumor related death (metastatic and/or local recurrence) or to the date of their last follow-up. Assessment of metastasis and recurrence of tumors was done by physical examination, Cytology (fine needle aspiration), and 3-dimentional thoracic radiograph by referral animal hospital and/or Seoul National University Veterinary Medical Teaching Hospital.

7. Statistical method

The $\chi 2$ test was used to detect the association between the FOXP3 expression and the characteristics of the dogs (such as their tumor size, lymph node status, and distant metastasis), histological parameters and immunohistochemical results. The Kaplan-Meier method and the Log-Rank test were used to construct survival curves for the dogs with differences in their expression of ERα, PR, HER2, and FOXP3. All the analyses were performed using commercial statistics software (SPSS, version 18.0, SPSS). $P < 0.05$ was considered significant for all the analyses.

III. Results

1. Dogs

All the 62 dogs were included in this study: 17 Yorkshire terriers (27.4 %), 15 Maltese (24.2 %), 7 mixed breeds (11.3%), 6 poodles (9.7%), and other breeds (27.4%), including Cocker spaniels, Shih-Tzus, Chihuahuas, and Schnauzers. Their age at the time of the surgical tumor resectioning ranged from 5 to 16 years (mean ± SD: 10.5 ± 3.0 years; median: 10.0 years). Their body weight ranged from 1.2 to 27.0 kg (mean ± SD: 4.7 ± 3.5 kg; median: 4.1 kg). Most of tumor size were less than 3 cm (n = 33, 53.2%). OHE was performed in 13 dogs (21.0%) before the tumor development. Inflammation around the tumor tissue was present in 34 dogs (54.8%). Tumor metastatic lesions were found at lymph node (3/62, 4.8%) and distant organs (2/62, 3.2%).

2. Histological and immunohistochemical analysis results

Of the 62 dogs, 40 had benign mammary gland tumors, which included 29 adenomas (72.5%) and 11 benign mixed tumors (27.5%). Twenty two dogs had malignant mammary gland tumors, which included 12 adenocarcinomas (54.5%) and 10 malignant mixed tumors (45.5%) (Table 3).

Table 3. Immunohistochemical results in 40 benign and 22 malignant mammary gland tumors of dogs.

Tumor Type	No. of Tumors	ERα+		PR+		HER2+		FOXP3+	
		No.	%	No.	%	No.	%	No.	%
Benign									
Adenoma	29	25	86.2	27	93.1	2	6.9	2	6.9
Benign mixed tumor	11	10	90.9	10	90.9	0	0.0	0	0.0
Total	**40**	**35**	**87.5**	**37**	**92.5**	**2**	**5.0**	**2**	**5.0**
Malignant									
Adenocarcinoma	12	2	16.7	1	8.3	2	16.7	6	50.0
Malignant mixed tumor	10	2	20.0	3	30.0	2	20.0	6	60.0
Total	**22**	**4**	**18.2**	**4**	**18.2**	**4**	**18.2**	**12**	**54.5**

ERα expression was observed in 35 samples (87.5 %) of total benign mammary gland tumours, which included 25 adenomas (86.2 %) and 10 benign mixed tumours (90.9 %). Whereas, only 4 samples (18.2 %) of total malignant mammary gland tumours, which included 2 adenocarcinomas (16.7 %) and 2 malignant mixed tumours (20.2 %).

PR expression was observed in 37 samples (92.5 %) of total benign mammary gland tumours, which included 27 adenomas (93.1 %) and 10 benign mixed tumours (90.9 %). But, only 4 samples (18.2 %) of total malignant mammary gland tumours, which included one adenocarcinoma (8.3 %) and 3 malignant mixed tumours (30.0 %).

HER2 over-expression was observed in 2 samples (5.0 %) of total benign mammary gland tumours, which only expressed in adenomas. And 4 samples (18.2 %) of total malignant mammary gland tumours, which included 2 adenocarcinomas (16.7 %) and 2 malignant mixed tumours (20.0 %).

The FOXP3 expression was variable in the canine mammary gland tumors. FOXP3 expression was observed in ≥ 25% of neoplastic cells in 2/40 (5.0%) benign mammary gland tumors and 12/22 (54.5%) malignant mammary gland tumors, whereas 38/40 (95.0%) benign mammary gland tumors and 10/22 (45.5%) malignant mammary gland tumors showed no staining or staining in < 25% of neoplastic cells (Table 3, Fig. 1A, B, and C).

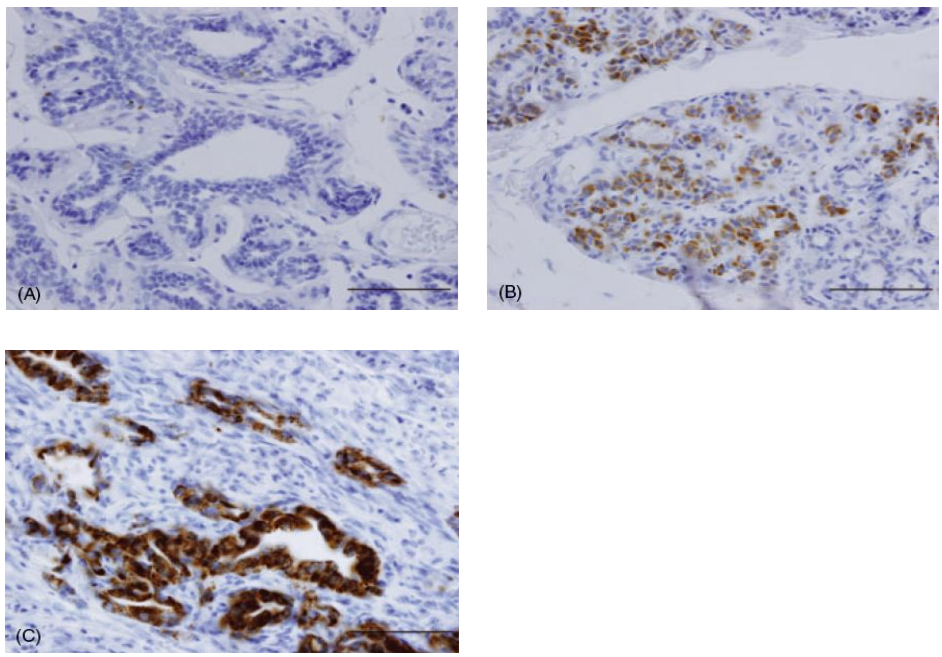

Fig. 1. Immunohistochemical expression of FOXP3 in canine mammary tumor tissue (magnification x 400; bar = 80 μm). (A) Benign mammary gland tumor, showing negative FOXP3 expression. (B) Benign mammary gland tumor, showing weak positive staining. (C) Malignant mammary gland tumor, showing strong positive staining.

Histological grade and subtypes among the malignant mammary gland tumors were analyzed. Three well-differentiated tumors (13.7%) versus 19 poorly-differentiated tumors (86.3%), 3 simple tumors (13.7%) versus 19 complex tumors (86.3%), and 2 solid tumors (9.1%) versus 20 tubulopapillary tumors (90.9%) (Table 4).

Table 4. Histological grade and subtypes of 22 canine malignant mammary gland tumours.

Type of Malignant tumors	No. of Tumors	ERα+		PR+		HER2+		FOXP3+	
		No.	%	No.	%	No.	%	No.	%
Histological grade									
Well-differentiated	3	0	0.0	0	0.0	1	33.3	2	66.7
Poorly-differentiated	19	4	21.1	4	21.1	3	15.8	10	52.6
Histological subtype									
Simple	3	0	0.0	0	0.0	1	33.3	2	66.7
Complex	19	4	21.1	4	21.1	3	15.8	10	52.6
Histological subtype									
Solid	2	0	0.0	0	0.0	0	0.0	2	100.0
Tubulopapillary	20	4	20.0	4	20.0	4	20.0	10	50.0

Tables 5 show the relationship between the FOXP3 expression and several clinicopathologic parameters. The positive and negative expressions of FOXP3 differed significantly with tumor size ($P = 0.025$), presence of inflammation ($P = 0.043$), and histological type ($P < 0.001$). Increased in tumour size has significant relations with negative expression of FOXP3. Especially, groups between tumours less than 3 cm and larger than 5 cm. 30/33 (90.9 %) tumours less than 3 cm shows negative FOXP3 expression. But, 7/11 (63.6 %) with tumours large than 5 cm.

Relationship between presence of inflammation and FOXP3 expression was found, 11/34 (32.4 %) tumour with inflammation shows positive FOXP3 expression whereas only 3/28 (10.7 %) tumours without inflammation shows positive FOXP3 expression

Histological type has significant correlations with FOXP3 expression. 38/40 (95.5 %) benign mammary gland tumours shows negative FOXP3 expression whereas 10/22 (45.5 %) in malignant mammary gland tumours.

Table 5. FOXP3 expression and clinicopathologic parameters.

Clinicopathologic parameters	No.	FOXP3 expression	
		Negative	Positive
Clinical stage	62		
I, II, or III	57	45 (78.9%)	12 (21.0%)
IV or V	5	3 (60.0%)	2 (40.0%)
P		*0.33*	
Tumor size (T)	62		
≤ 3 cm	33	30 (90.9%)	3 (9.1%)
3 - 5 cm	18	11 (61.1%)	7 (38.9%)
〉 5 cm	11	7 (63.6%)	4 (36.4%)
P		*0.025*	
Lymph node metastasis (N)	62		
Absent	59	46 (78.0%)	13 (22.0%)
Present	3	2 (66.7%)	1 (33.3%)
P			
Distant metastasis (M)	62		
Absent	60	47 (78.3%)	13 (21.7%)
Present	2	1 (50.0%)	1 (50.0%)
P		*0.34*	

Table 5. FOXP3 expression and clinicopathologic parameters (Continued).

Clinicopathologic parameters	No.	FOXP3 expression	
		Negative	Positive
Inflammation	62		
Absent	28	25 (89.3%)	3 (10.7%)
Present	34	23 (67.6%)	11 (32.4%)
P		*0.043*	
Histological type	62		
Benign	40	38 (95.0%)	2 (5.0 %)
Malignant	22	10 (45.5 %)	12 (54.5 %)
P		*<0.001*	
Age	62		
≤ 9 years old	27	22 (81.5%)	5 (18.5%)
〉 9 years old	35	26 (74.3%)	9 (25.7%)
P		*0.5*	
Breed	62		
Yorkshire terriers	17	16 (94.1%)	1 (5.9%)
Maltese	15	9 (60.0%)	6 (40.0%)
Mixed breed	7	7 (100.0%)	0 (0.0%)
Poodles	6	4 (66.7%)	2 (33.3%)
Others	17	12 (70.6%)	5 (29.4%)
P		*0.084*	

Table 6 shows the associations between the FOXP3 expression and the other molecular markers. The FOXP3 expression significantly differed from the staining groups for ERα and PR. The tumors with negative FOXP3 staining were ERα positive (38/48, 79.2%) ($P < 0.001$) and PR positive (40/48, 83.3%) ($P < 0.001$). HER2 overexpression was observed 6/62 (9.7%) of total tumor samples, and 4/22 (18.2%) of malignant tumor samples. But, there was no significant correlationship between HER2 overexpression and FOXP3 expression.

Table 6. FOXP3 expression and other molecular markers.

Molecular markers	No.	FOXP3	
		Negative	Positive
ERα	62		
Negative	23	10 (43.5%)	13 (56.5%)
Positive	39	38 (97.4%)	1 (2.6%)
P		*<0.001*	
PR	62		
Negative	20	8 (40.0%)	12 (60.0%)
Positive	42	40 (95.2%)	2 (4.8%)
P		*<0.001*	
HER2	62		
Negative	56	44 (78.6%)	12 (21.4%)
Positive	6	4 (66.7%)	2 (33.3%)
P		*0.51*	

3. Prognostic significance of the FOXP3 expression

All the dogs followed post-surgically had a median DFS time of 15 months (range: 2-54 months). Fourteen dogs died due to the progression of the disease (metastatic and/or local recurrence). The Kaplan-Meier survival curves of tumor related death showed negative FOXP3 expression in canine mammary gland tumors, and this was associated with a longer DFS (Fig. 2). A multivariate analysis was performed to investigate whether or not FOXP3 expression is an independent prognostic factor of canine mammary gland tumors. The data showed, however, that FOXP3 expression is not an independent prognostic factor.

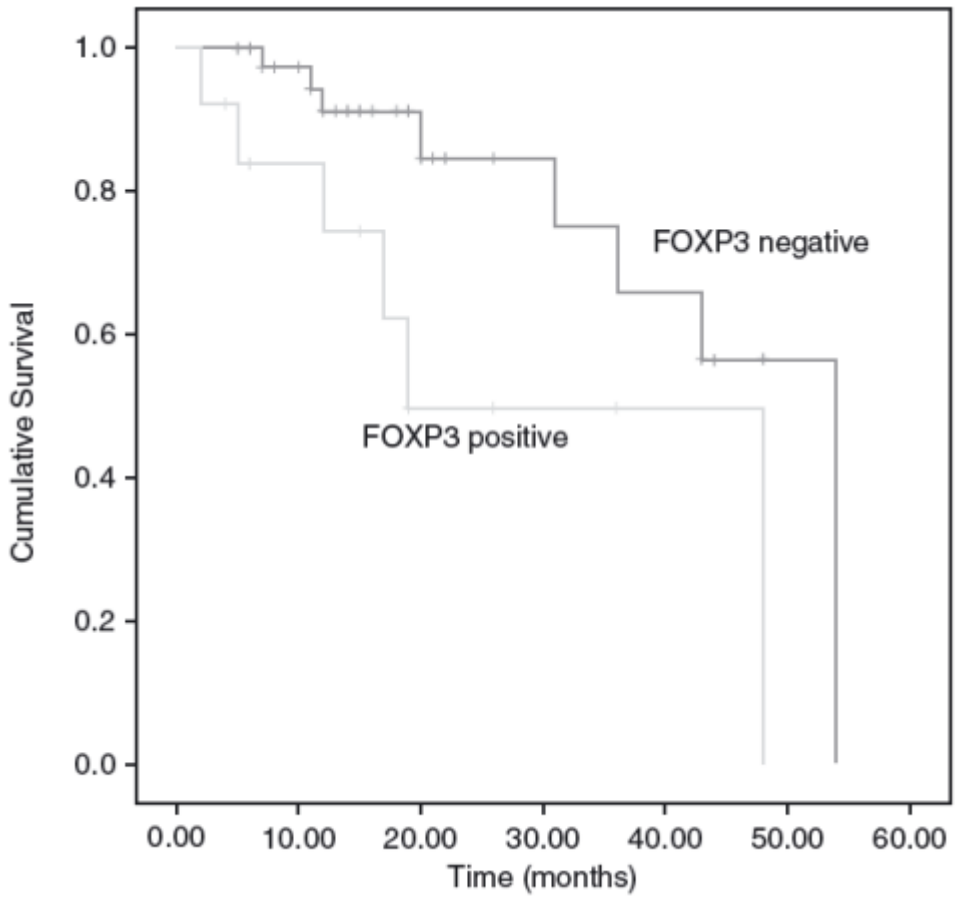

Fig. 2. Kaplan-Meier disease free survival curves of groups with positive and negative FOXP3 expression. Dogs with mammary gland tumor (*P* = 0.029).

IV. Discussion

Nearly half of mammary tumors that occur in bitches are considered malignant in the canine species (Gilbertson et al. 1983). Histologic malignancy does not always imply a malignant clinical course, though, and a marked variation in the histologic appearance can occur within the same tumor mass (J.Withrow and Vail 2007). It is important to determine reliable prognostic factors of individual risk of an unfavorable clinical outcome. Recently, FOXP3 expression has been identified as an independent prognostic factor in human breast cancer (Merlo, Casalini et al. 2009). This study demonstrates that patterns of FOXP3 expression in canine mammary gland tumors differ from those of human breast carcinoma but may have a biological role in canine mammary gland tumors, although they are not an independent prognostic factor.

In human research, immunohistochemical studies have shown the expression of FOXP3 in tumor cells in ovarian (Curiel, Coukos et al. 2004), liver (Ormandy, Hillemann et al. 2005), gastric, and esophageal cancer (Ichihara, Kono et al. 2003). In human breast cancer, FOXP3 is expressed in breast cancer cells, and the expression level is associated with patient survival (Merlo, Casalini et al. 2009). To the best of the authors' knowledge,

this is the first study that correlates the expression of FOXP3 with clinical and pathological parameters and survival for canine mammary gland tumors.

The patterns of FOXP3 expression were investigated via immunohistochemistry in 62 canine mammary gland tumors. In this study, the staining of neoplastic cells was heterogeneous, and ranged from mostly cytoplasmic to both cytoplasmic and nuclear. This staining heterogeneity is consistent with the observations in human breast cancer research (Merlo, Casalini et al. 2009). Approximately 22.6% of canine mammary gland tumors were scored positive for FOXP3 expression. Malignant mammary gland tumors showed more significant FOXP3 expression (12/22, 54.5%) than benign mammary gland tumors (2/40, 5.0%). However, histological grade and subtype among the malignant mammary gland tumor showed no correlations with FOXP3 expression (Table 2).

FOXP3 expression was found to be correlated with large tumor size ($P < 0.025$), presence of inflammation ($P < 0.043$) and tumor malignancy ($P < 0.001$), but not with other clinicopathologic parameters such as age, breed, clinical stage, lymph node metastasis and distant metastasis. Significant correlations between inflammation and FOXP3 expression in this research may be due to Tregs function as mediators of immune evasion mechanisms. As

previously mentioned, FOXP3 is marker for immunosuppressive Tregs. Tregs have role in autoimmune and infectious disease. Tregs also have been shown to be important in the body's response to tumor. In human research, higher numbers of Tregs have been found in the peripheral blood and neoplastic tissues of patients with a variety of tumors, including breast carcinoma (Bates et al. 2006), ovarian carcinoma (Curiel, Coukos et al. 2004), gastric carcinoma (Ichihara, Kono et al. 2003), melanoma (Viguier et al. 2004), and others. Also in veterinary research, Treg numbers were significantly higher in peripheral blood of dogs with cancer than healthy dogs (Biller, Elmslie et al. 2007). In addition, it appears that certain types of tumors in dogs, especially highly malignant tumors, may be associated with higher numbers of Tregs. Some research suggest that Tregs may play a role in inducing immune tolerance to higher grade, hormone receptor negative breast carcinomas, which Tregs are associated with more aggressive breast cancer phenotype (Bohling and Allison 2008). FOXP3 expression in mammary gland tumors may suggest abundance of Tregs which means increased immune tolerance to mammary gland tumors. Consequently, FOXP3 induced immune tolerance may affect aggressive tumor growth which lead inflammation around the tumor tissue. In human study, FOXP3 expression has significant correlation with lymph node metastasis(Merlo, Casalini et al. 2009). This result counter to our present study. But, this maybe due to absolute shortage of cases which makes it difficult to make a comparison betweem human and our study.

The FOXP3 positive tumors in this study were associated with negative expression of ERα and PR ($P < 0.001$), which are poor prognostic factors of canine mammary gland tumor (Misdorp and Hart 1976; Sartin et al. 1992; Chang et al. 2009). These findings may suggest that FOXP3 expression is more likely to occur in malignant mammary gland tumors than in benign mammary gland tumors. There was no correlation, however, between FOXP3 and HER2 overexpression. Immunohistochemical overexpression of HER2 in canine mammary gland tumor has variable result. 19.4% (Rungsipipat, Tateyama et al. 1999) and 74% (Ahern, Bird et al. 1996). This is also same result from human study. Maybe this large range result is due to the number of cases or to the different immunohistochemical method. In our study, HER2 overexpression was 6/62 (9.7%) which is lower than previous reports. But, HER2 expression in malignant mammary gland tumor was 4/22 (18.2%) which is corresponsive to later reports. Therefore, it seems to be necessary to standardize the methods to obtain objective result for HER2 overexpression.

The clinical follow-up in this study indicated a significant correlation between FOXP3 and the decreased DFS from canine mammary gland tumors ($P = 0.029$), which is similar to the results of human breast cancer research (Merlo, Casalini et al. 2009). However, considering this human study which only include malignant breast cancer, our study include both benign and malignant mammary gland tumors which makes it difficult to conclude

significance of FOXP3 expression and overall survival in canine mammary gland tumor. But, in this study we evaluate correlation of FOXP3 expression with Benign and malignant tumor. We found that there were correlations of FOXP3 negative with benign mammary gland tumors and Hormonal receptor expression. This result may suggest the potentials that FOXP3 negative have high prediction rate of benign mammary gland tumor. Furthermore, FOXP3 expression and survival curve graph that based on tumor related death may provide evidence for correlations between FOXP3 negative and benign mammary gland tumor. We also think that this result can be a basic data used for further studies to evaluate FOXP3 expression and overall survival in canine malignant mammary gland tumors.

In this study, we evaluate the immunohistochemical expression for FOXP3 in canine mammary gland tumor, and its relationship with some other known prognostic factors. Our findings demonstrate that negative expression of FOXP3 has high prediction rate for benign mammary gland tumor, and significant correlation with hormonal receptors expression. We also found some relationship between FOXP3 expression and tumor related death. Further studies including a large number of malignant mammary gland tumors are needed to confirm the FOXP3 expression as a prognostic factor in canine mammary gland tumors.

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VI. 국문초록

개 유선 종양에서의 FOXP3 발현 평가 연구

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본 연구는 최근 인의에서 유방암의 발생과 생존율에 영향을 미치는 예후인자로 보고되고 있는 전사인자 FOXP3 (forkhead box P3)에 대하여, 개의 유선종양에서의 발현 여부를 평가하였다. 또한, 개의 유선종양에 있어서 기존의 다른 예후인자들과 FOXP3의 발현을 비교하고, 생존율과의 연관성을 확인해 보았다.

62마리의 개에서 전사인자 FOXP3의 발현을 면역조직화학염색 분석을 통하여 평가하고, 기존의 다른 예후인자들과의 연관성 및 생존율을 분석하였다.

연구에 사용된 유선종양의 22.6 %에서 FOXP3 양성 발현이 확인되었으며, 이는 종양의 크기 (P=0.025), 염증 (P=0.043), 그리고 조직학적인 악성도

(P<0.001)와의 연관성이 확인되었다. FOXP3 음성의 경우, 호르몬 수용체 (Estrogen receptor-α, Progesterone receptor)의 양성 발현과의 연관성이 확인되었다 (P<0.001). 또한, 개의 유선종양에 있어서 FOXP3의 발현은 무병생존기간 (Disease free survival)과의 유의적인 연관성이 확인되었다 (P=0.029).

이상의 결과를 토대로 FOXP3 음성인 유선종양의 경우, 양성 유선종양에 대한 높은 예측률을 가지고 있으며, 유선종양에서의 호르몬 수용체 양성 발현과 유의적인 상관관계가 있다는 것을 알 수 있다. 또한, FOXP3 양성 발현과 무병생존기간의 단축과의 유의적인 관계를 확인할 수 있었다.

Keywords: 개, FOXP3, 면역조직화학염색, 유선종양, 예후인자
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