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RESEARCH ARTICLE

Evaluation of Tumor Prognostic Markers in Malignant Canine Mammary Carcinoma

HW Kim[1], YG Jang[2], JI Shin[1], BJ Seung[1], JH Ju[1], SM Lee[2] and JH Sur[1]*

[1]Department of Veterinary Pathology, Small Animal Tumor Diagnostic Center; [2]Department of Veterinary Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Korea
*Corresponding author: jsur@konkuk.ac.kr

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A B S T R A C T

Tumor malignancy is closely associated with cancer prognosis. However, each malignant tumor can have considerably different prognosis. Therefore, we aimed to characterize the factors that increase the malignancy of tumors by analyzing the associations between factors associated with tumor malignancy and prognosis and histological parameters of tumor malignancy. Ki-67, p53, p63, bcl-2, UGT-8 antibody expression was detected in malignant tumor of canine by immunohistochemistry. The results showed expression of Ki-67 was associated with histological subtypes of tumor, histological grade, and lymphatic invasion ($P<0.05$). Expression of p53 in the nucleus was only shown in inflammatory carcinoma types and significantly associated with histological type, high histological grade, and lymphatic invasion ($P<0.05$). High expression of p53 including cytoplasmic expression was shown to be negatively associated with central necrosis and lymphocyte infiltration ($P<0.05$). Expression of p63 was associated with molecular subtype, and Bcl-2 was shown to be associated only with central necrosis in tumors ($P<0.05$). But no relationship was found between UGT-8 expression and histopatholgical characteristics in malignant canine mammary tumors. In the canine malignant mammary tumors, we found that Ki-67 can be used as a favorable prognostic marker. But other markers or indices were not useful in evaluating malignancy of tumors.

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INTRODUCTION

With the growing number of deaths due to cancer, many studies have been conducted to better understand the disease. To understand and treat cancer properly, we need to understand tumor development and progression in advance. In cancer, the growth rates of tumor cells and tumor invasiveness are elevated. Occasionally, metastasis via lymphatic or vascular pathways occur. In malignant tumor, it is not the tumor itself that is the primary cause of death; rather, in breast cancer, metastasis is the most important cause of death (Greenberg *et al*., 1996).

There are many factors associated with poor prognosis and tumor malignancy. Sex steroid hormones such as estrogen and progesterone are associated with breast cancer development and progression due to activated hormone receptors (Murphy and Watson, 2002). The estrogen receptor (ER) and progesterone receptor (PR), along with the human epidermal growth factor receptor-2 (HER-2), can be used in the molecular-based classification of mammary cancer in both women and female dogs (Gama *et al*., 2008). According to this classification, specific receptor pathway-dependent determination of prognosis and treatment of cancer is performed (Sorenmo *et al*., 2011).

Several studies have found that highly proliferative tumor cells are associated with poor prognosis (Haerslev *et al*., 1996; Brown and Gatter, 2002). To evaluate the tumor cell growth rate, cell cycle-related markers are commonly used. Ki-67 is known to be a good marker of a cellular proliferation and has prognostic value in canine mammary tumors (Brown and Gatter, 2002).

The p53 is a well-known tumor suppressor gene. Mutations in the p53 gene are characteristic features of most tumors, and can be used as an independent prognostic factor in most human cancers and canine mammary tumors (Lee *et al*., 2004). The p63 gene serves as a suppressor of tumorigenesis by interacting with mutant p53, which is associated with tumor invasion and metastasis (Barbieri *et al*., 2006). However, the exact role

of p63 in tumors has not been fully determined. As a basal marker, the p63 gene is known to be associated with epithelial to mesenchymal transition (EMT) (Sarrió *et al*., 2008). EMT plays an important role in carcinoma prognosis and affects the migratory behavior of cancer cells (Sarrió *et al*., 2008).

The UGT-8 is a molecular marker of breast cancer malignancy (Dzi *et al*., 2010) and is associated with breast cancer lung metastasis (Landemaine *et al*., 2008). An association between UGT-8 expression in canine mammary tumors and tumor malignancy has also been reported (Nowak *et al*., 2013).

Another tumor marker, Bcl-2, is also recognized as a prognostic marker of breast cancer (Callagy *et al*., 2006). Bcl-2 is included and analyzed with Ki-67 for the Ki-67/Bcl-2 index. The prognostic value of the Ki-67/Bcl-2 index in breast cancer has been proposed by others (Ali *et al*., 2012).

In this study, disease prognosis and tumor cell malignancy-associated factors were evaluated to characterize malignant canine mammary tumors. Histological classification, histological grading, lymphatic invasion, central necrosis, and lymphatic infiltration were evaluated. In addition, the expression of markers related to tumor progression and prognosis was assessed by immunohistochemistry, and its relationship with the outcomes was analyzed.

MATERIALS AND METHODS

Sample selection: Primary canine mammary tumor samples banked by the Department of Veterinary Pathology, Konkuk University Animal Teaching Hospital, Seoul, Korea were analyzed. From the total samples, 45 malignant carcinomas with available necessary information were randomly selected. All samples were fixed in 10% buffered neutral buffered formalin and embedded in paraffin wax.

Tumor classification: Sections with 4 µm thickness were stained with hematoxylin and eosin (H&E) for histological evaluation. Histological classification was performed on H&E-stained slides, based on the formerly proposed criteria (Goldschmidt *et al*., 2011). Histological grading was assessed as well-differentiated tumor (grade 1), moderately differentiated tumor (grade 2), poorly differentiated tumor (grade3) as proposed by Peña *et al*. (2013).

Immunohistochemistry: Sections (4 µm thick) of formalin-fixed paraffin-embedded tissues were deparaffinized by xylene, serial rehydrated in graded ethanol, and three times washed in phosphate buffered saline (PBS). The PBS-diluted 3% hydrogen peroxide was used to block endogenous peroxidase activity. After PBS washing, antigen retrieval was performed by the microwave retrieval method. Microwave (750 W, 60 Hz, 15 min) retrieval in pH 9.0 Tris-EDTA buffer was used for the anti-ER-, PR-, p63-, and UGT-8-staining samples and in pH 6.0 citric acid buffer was used for anti- HER-2, Ki67, and p53 staining samples. Thereafter, 5% normal goat serum was incubated on plates for 30 min for anti-ER-, Bcl-2-, and UGT-8-staining samples to block nonspecific binding. The primary antibody was applied without PBS washing. For anti-ER (diluted 1:60 in PBS) antibody staining, plates were incubated 3 h at room temperature; for anti-PR (1:500), HER-2/neu (1:300), Ki67 (1:300), p53 (1:200), p63 (1:100), Bcl-2 (1:300), and UGT-8 (1:200) antibody staining, plates were incubated overnight at 4°C. After a PBS washing, immunolabeling with secondary antibody-conjugated HRP was performed with 40 min incubation. Immuno-labeled antigens were visualized using DAB+ chromogen, washing with distilled water, counterstaining with Gill's hematoxylin, and adding coverslips to slides were conducted in sequence.

Immunohistochemical evaluation: Nuclear ER and PR expression was considered positive if there was more than 10% expression on tumor cells (Gama *et al*., 2008). Evaluation of HER-2 expression was based on the Hercep test, and more than 10% of complete plasma membrane expression was considered positive (Kim *et al*., 2013).

To evaluate Ki-67 expression, the percentage of cells with nuclear reactivity were estimated by counting. Averaged percentages of Ki-67 expression were acquired at least 3 representative 200X magnified fields, the proportion of Ki-67 expression was scored into three categories: 0, 0~10%; 1, 11~32%; 2, >33%.

The p53 expression is commonly considered positive only with nuclear staining. However, in this study, not only nuclear expression, but also strong cytoplasmic expression for p53 staining was considered positive. The p63 expression in a neoplastic cell was considered positive, but additional analyses, including that for expression in the surrounding myoepithelium, were performed separately. With both processes, more than 10% of expression was considered positive.

More than 10% moderate or high expression of Bcl-2 in a cell was considered to indicate positivity. Assessment of UGT-8 was performed with the Immunoreactive Remmele Scale (IRS) (Remmele and Stegner, 1987).

To apply the Ki-67/Bcl-2 index, staining intensity of Bcl-2 was scored into three categories: 0, none/low; 1, moderate; 2, high. By subtracting the Bcl-2 score from the Ki-67 score, the values were divided into 3 categories: -2=low risk; 1 or 0=intermediate risk; 1 or 2=high risk (Ali *et al*., 2012). Then, associations with other factors were analyzed.

Statistical analysis: Pearson's chi-squared test or Fisher's exact test was performed to determine statistical significance of any association. The statistical significance level was defined as $P<0.05$. All statistical analyses were performed using The Statistical Package for Social Science software for Windows, version 17.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Histopathological characteristics: A total of 45 samples with malignant canine carcinoma were analyzed in this study. Histological classifications included epithelial neoplasms (n=19), mixed tumors (n=21), and special types (n=5). In more detail, histological classifications included carcinomas arising in a complex adenoma/mixed tumor (n=20; Fig. 1A), complex carcinoma (n=1; Fig. B1),

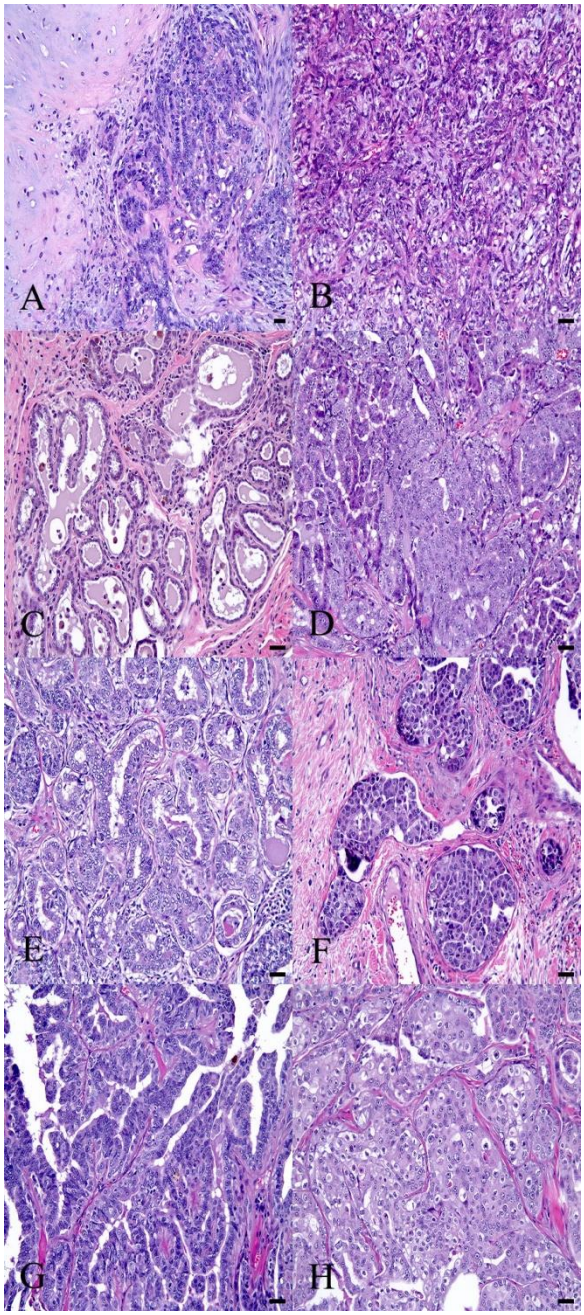

Fig. 1: Histopathological features of canine mammary tumor. A) Carcinoma arising in a complex adenoma/ mixed tumor. B) Carcinoma–complex type. C) Carcinoma–simple type. D) Carcinoma–solid type. E) Ductal carcinoma. F) Inflammatory carcinoma. G) Intraductal papillary carcinoma. H) Squamous cell carcinoma. H&E stain. Bar=35 μm.

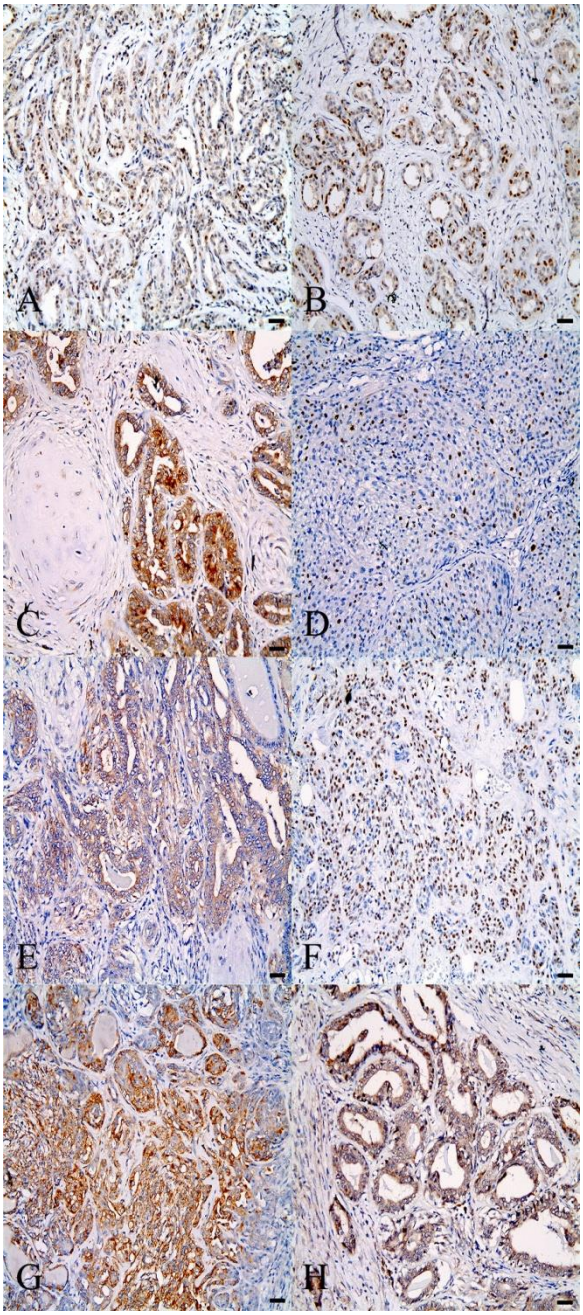

Fig. 2: Immunohistochemical results. Positive expressions of A) estrogen receptor, B) progesterone receptor, C) human epidermal growth factor receptor-2, D) Ki-67, E) p53, D) p63, E) Bcl-2 F) UGT-8 staining in canine mammary carcinomas. Peroxidase/DAB; Gill's hematoxylin counterstain. Bar=35 μm.

ductal carcinoma (n=4; Fig. 1C), simple carcinoma (n=3; Figure 1D), solid carcinoma (n=7; Figure 1E), intraductal papillary carcinoma (n=5; Figure 1F), inflammatory carcinoma (n=2; Figure 1G), and mammary squamous cell carcinoma (n=3; Figure 1H). On histological grade assessment, 29 tumors were grade 1, 10 were grade 2, and 6 were grade 3. Of all samples, 20.0% (n=9) exhibited lymphatic invasion, and 51.1% (n=23) demonstrated central necrosis.

Evaluation of Immunohistochemical results: Among all samples, 28.9% (n=13; Figure 2A) were ER positive, 64.4% (n=29; Figure 2B) were PR positive, and 73.3% (n=33; Figure 2C) were HER-2 positive. In the molecular classification of tumors, types of tumors that are positive for hormone receptor expression are called luminal types. Luminal A type (positive expression for ER or PR, and negative expression for HER-2) comprised 13.3% (n=6) of the total samples, while luminal B type (positive expression for ER or PR, and negative expression for HER-2) represented 64.4% (n=29) of samples. Types of tumors that were hormone receptor negative but were positive for HER-2 receptor expression, HER-2-overexpressing type, accounted for 8.9% (n=4) of samples. Tumors that were negative for the expression of all three receptors, triple negative type, included 13.3% (n=6) of the total.

Table 1: Correlations between histopathological features of mammary carcinomas with Ki-67 expression

		Ki-67 (%)			P
		0 (n=27)	1 (n=7)	2 (n=11)	
Histological classification	Carcinoma arising in a complex adenoma/mixed tumor	16(80.0)	3(15.0)	1(5.0)	0.002*
	Carcinoma- complex type	1(100.0)	0(0.0)	0(0.0)	
	Carcinoma- simple	2(66.7)	1(33.3)	0(0.0)	
	Carcinoma- solid type	1(14.3)	2(28.6)	4(57.1)	
	Intraductal papillary carcinoma	2(40.0)	0(0.0)	3(60.0)	
	Ductal carcinoma	3(75.0)	1(25.0)	0(0.0)	
	Inflammatory carcinoma	2(100.0)	0(0.0)	0(0.0)	
	Squamous cell carcinoma	0(0.0)	0(0.0)	3(100.0)	
Histological grade	1	23(79.3)	4(13.8)	2(6.9)	0.000*
	2	1(10.0)	3(30.0)	6(60.0)	
	3	3(50.0)	0(0.0)	3(50.0)	
Lymphatic invasion	Absent	25(69.4)	4(11.1)	7(19.4)	0.020*
	Present	2(22.2)	3(33.3)	4(44.4)	

Category 0 = 0-10%, Category 1 = 11-32%, Category 2 = >33%, statistical significance; P<0.05; * Fisher's exact test

Table 2: Correlations between histopathological features of mammary carcinomas with p53 expression

		P53[1] (%)		P
		Negative (n=26)	Positive (n=19)	
Central necrosis	Absent	9(40.9)	13(59.1)	0.026
	Present	17(73.9)	6(26.1)	
Lymphocyte infiltration	Absent	0(0.0)	5(100.0)	0.010*
	Present	26(65.0)	14(35.0)	

p53 [1]= high p53 expression, including cytoplasmic expression, statistical significance; P<0.05; * Fisher's exact test

Table 3: Correlations between histopathological features of mammary carcinomas with p63 expression

		P63 (%)		P
		Negative (n=10)	Positive (n=35)	
Molecular subtype	Luminal A	1(16.7)	5(83.3)	0.007*
	Luminal B	3(10.3)	26(89.7)	
	HER-2 overexpressing	3(75.0)	1(25.0)	
	Triple negative	3(50.0)	3(50.0)	

p63; >10% positivity of nuclear p63 expression, Luminal A; ER+ or PR+/HER-2-, Luminal B; ER+ or PR+/HER-2+, HER-2 overexpressing; ER-/PR-/HER-2+, Triple negative; ER-/PR-/HER-2-, statistical significance; P<0.05; * Fisher's exact test

Table 4: Correlations between histopathological features of mammary carcinomas with Bcl-2 expression

		Bcl-2 (%)		P
		Negative (n=14)	Positive (n=31)	
Central necrosis	Absent	10(45.5)	12(54.5)	0.043
	Present	4(17.4)	19(82.6)	

Statistical significance; P<0.05.

The Ki-67 showed a nuclear staining pattern (Figure 2D). The number of positive cells were counted and categorized into 3 scores. Samples scoring 0 were 60% (n=27) of the total, while those scoring 1 were 15.6% (n=7) and 2 were 24.4% (n=11).

Of tumors, 4.4% (n=2) were p53 positive when evaluated by nuclear expression, while it was 42.2% when high cytoplasmic expression was also considered positive (Figure 2E).

Immunohistochemical staining for anti-p63 antibody showed nuclear expression (Figure 2F), but there were some distinct patterns. Samples with expression on neoplastic cells without myoepithelial staining were 15.6% (n=7) of the total samples, while total samples positive for p63 expression, including those positive by myoepithelial staining, were 77.8% (n=35) of the specimens. Most malignant tumors were Bcl-2 positive (Figure 2G). Of the total, 68.9% (n=31) were Bcl-2 positive and 31.1% (n=14) were Bcl-2 negative. All samples were positive for the expression of UGT-8 (Figure 2H). The Ki-67/Bcl-2 index, prognostic markers of breast cancers in humans, was divided into 8.9% in the low risk group (n=4), 68.9% in the intermediate risk group (n=31), and 22.2% in the high risk group (n=17.8). Most tumors (91.1%, 41) were shown to be intermediate to high risk in malignant canine mammary tumors.

Correlations of immunohistochemical results with clinicopathological parameters: The expression of Ki-67 was significantly associated with histological classifications. Low expression of Ki-67 was associated with carcinomas arising in a complex adenoma/mixed tumor, complex carcinoma, simple carcinoma, ductal carcinoma, and inflammatory carcinoma. High expression of Ki-67 was associated with solid carcinomas and squamous cell carcinomas. In the histological grade analysis, grade 1 was related to low expression of Ki-67. Absence from the lymphatics was also significantly associated with low expression of Ki-67 (Table 1).

Expression of p53 in the nucleus was only shown in inflammatory carcinoma types of canine mammary tumors. But high expression of p53, including cytoplasmic expression, was shown to be negatively associated with central necrosis and lymphocyte infiltration. Tumors that showed central necrosis and lymphocyte infiltration tended not to express p53, while tumors that did not show central necrosis and lymphocyte infiltration tended to express p53 (Table 2).

Expression of p63 was associated with sex hormone positivity. In the molecular subtype analysis of tumors, luminal A and B types were closely associated with p63 expression, while other parameters did not show significant associations with these tumor types (Table 3). Tumors with central necrosis tended to express Bcl-2 (Table 4). On evaluation with the IRS scale, no relationship was found between UGT-8 and other factors in malignant canine mammary tumors.

In the Ki-67/Bcl-2 index analysis, most tumors that were associated with intermediate and high risk group, and low risk were found to be classified histologically as carcinomas arising in a complex adenoma/mixed tumor and complex carcinoma type (Table 5).

Table 5: Correlations between histopathological features of mammary carcinomas with Ki-67/Bcl-2 index

		Ki-67/Bcl-2 index (%)			P
		Low risk (n=4)	Intermediate risk (n=31)	High risk (n=10)	
Histological classification	Carcinoma arising in a complex adenoma/mixed tumor	3(15.0)	15(75.0)	2(10.0)	0.030*
	Carcinoma- complex type	1(100.0)	0(0.0)	0(0.0)	
	Carcinoma- simple	0(0.0)	3(100.0)	0(0.0)	
	Carcinoma- solid type	0(0.0)	4(57.1)	3(42.9)	
	Intraductal papillary carcinoma	0(0.0)	2(40.0)	3(60.0)	
	Ductal carcinoma	0(0.0)	4(100.0)	0(0.0)	
	Inflammatory carcinoma	0(0.0)	2(100.0)	0(0.0)	
	Squamous cell carcinoma	0(0.0)	1(33.3)	2(66.7)	

Statistical significance; P<0.05; * Fisher's exact test.

Table 6: Correlations between Ki-67 expression and p63 expression

		Ki-67 (%)			P
		0 (n=27)	1 (n=7)	2 (n=11)	
P63	Positive	26	5	7	0.021
	Negative	1	2	4	

Category 0 = 0-10%, Category 1 = 11-32%, Category 2 = >33%; statistical significance; P<0.05.

DISCUSSION

The prognosis of a tumor depends on its virulence as well as distant metastasis (Galea *et al*., 1992). Virulence is determined by malignancy characteristics of the tumor, and one of the criteria determining tumor malignancy and poor prognosis is the growth rates of tumor cells (Haerslev *et al*., 1996). A tendency to proliferate reflects the rapidly growing characteristic of the tumor; it is often represented by mitotic figures in the histological analysis.

The Ki-67 is a nuclear protein that is present in the active phases of the cell cycle. Therefore, it can be used as a cell proliferation marker (Gerdes *et al*., 1986). The Ki-67, used in this study as a proliferation marker, was shown to be associated with malignancy of tumors. In the histological analysis, low expression of Ki-67 was associated with epithelial and mixed types of tumors. In detail, low expression of Ki-67 was associated with carcinoma arising in a mixed tumor and carcinoma-simple type that known to have better prognoses. A low tumor grade commonly indicates good prognosis. Low histological grades of tumor have also been shown to be significantly associated with low expression of Ki-67. Tumor cells of malignant tumors have the potential to metastasize, but low expression of Ki-67 has been associated with tumor cells that do not invade the lymphatic vessels. This means that tumors with low proliferation rates tend not to metastasize. We can infer from these results that some tumors, represented by low expression of Ki-67, have a slow growing tendency compared to other types of tumors, even malignant ones, and that this can indicate a favorable prognosis.

As one other criterion of histologically assessing tumor malignancy, lymphatic invasion is remarkable. Tumor cells can commonly metastasize via lymphatic pathways, and lymphatic invasion can be a clue of distance metastasis (Galea *et al*., 1992). As mentioned previously, metastasis of tumors is one of the important underlying causes of death from cancer (Greenberg *et al*., 1996). Therefore, lymphatic invasion is an important marker in expecting prognosis, as well as in determining tumor malignancy.

The p63 gene is known as a homologue of the p53 tumor suppressor gene (Yang and McKeon, 2000). The actual role of the p63 gene in tumors is not well known, but it is preferentially expressed on myoepithelial cells (Gama *et al*., 2003). There have been reports of the association of myoepithelial and stem or breast progenitor cells (Barbareschi *et al*., 2001). In the breast cancer stem cell hypothesis, cancer cells undergoing EMT show stem-like properties, and EMT has been closely related to aggressive and metastatic characteristics of tumors (Savagner, 2001; Thompson and Newgreen, 2005). Role of p63 in tumor metastasis has been reported in some studies (Barbieri *et al*., 2006). But in this study, no significant associations were found with p63 expression, except the association between Ki-67 and p63 expression. The positive expression of p63 was associated with low expression of Ki-67, and negative expression of p63 is related to high expression of Ki-67 (Table 6). Along with the previously described characteristics of Ki-67 expression with metastasis, the connection between Ki-67 and p63 seems considerable.

The UGT-8 is a gene that is highly associated with breast cancer lung metastasis (Landemaine *et al*., 2008), and that has also been associated with mammary tumor malignancy (Dzi *et al*., 2010; Nowak *et al*., 2013). As a marker of malignant canine mammary tumors, all samples were positive for UGT-8 immunohistochemical staining. However, when analyzed with the IRS scale, no relations with other parameters related to prognosis or lymphatic invasion were found among the malignant tumors in this study.

Although p63 and UGT-8 expressions were not shown to be critically associated with lymphatic invasion in this study, further investigations into the factors that are associated with tumor cell malignancy and tumor metastasis in malignant tumors, including other EMT associated markers, are needed.

Grade is the most classically used indicator of tumor prognosis. Generally, tumors with higher grade tend to show poor prognosis. In this study, not all tumors with high grades showed lymphatic invasion. But tumors with low grades rarely show lymphatic invasion and tend to show low mitotic rates and low expression of Ki-67. Additionally, low grade tumors show low Ki-67/Bcl-2 scores. Therefore, we can reaffirm that low grades at least suggest a better prognosis.

Malignant tumors are the tumors that are commonly expected to show poor prognosis. This study was conducted to estimate how prognosis-associated markers and malignancy-associated characteristics were associated in malignant canine mammary tumors. Among the malignant tumors, no markers or prognostic index, except for Ki-67, was found to be useful as an independent

prognostic indicator of malignancy characteristics in malignant tumors. Low Ki-67 expression in malignant tumor can be used as a favorable prognostic factor in tumors. Additionally, the association of a low proliferation rate of tumor cells and the lack of lymphatic invasions of tumor cells was found. This suggests that even with equally malignant tumor conditions, highly proliferating cells can attain the ability to invade lymphatic vessels by pathways that were not identified in this study.

Conclusions: Even in malignant tumor, different progression and prognosis are shown in malignant tumors by its malignancy. Among the analysis of the relations between prognosis-associated markers and malignancy-associated characteristics of the tumor, low expression rates of Ki-67 can be used as a favorable prognostic factors in malignant tumors.

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Author's contribution: HW Kim carried out most of the work. YG Jang, JI Shin participated in IHC and statistical analysis. BJ Seung, SM Lee and JH Ju participated in experimental design. JH Sur supervised in its design and helped to draft the manuscript. This report represents a part of the PhD thesis by YG Jang. All authors read and approved the final manuscript.

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