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ErbB2 copy number gain is associated with adverse outcome in canine mammary carcinoma

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ABSTRACT. Copy number gain (CNG) and/or protein overexpression of ErbB2 have been observed in human breast cancer patients and are associated with poor prognosis. Similarly, ErbB2 overexpression has also been observed in canine mammary carcinoma; however, data on *ErbB2* copy number is limited. The purposes of this study were to evaluate *ErbB2* copy number in dogs with mammary carcinoma and to investigate associations of *ErbB2* CNG with ErbB2 expression, histological and clinical characteristics, and survival. DNA samples were isolated from 59 formalin-fixed paraffin-embedded canine mammary gland tissues (34 carcinoma, 14 adenoma, and 11 normal). Using a digital PCR assay, the *ErbB2* copy number in these samples was determined as compared to a reference gene on canine chromosome 8. *ErbB2* CNG was detected in 14/34 (41%) carcinomas and 2/14 (14%) adenomas. ErbB2 overexpression was observed in 3/34 (9%) carcinomas but not in adenomas. Neither *ErbB2* CNG nor ErbB2 overexpression were detected in the normal controls. There was no significant association of the *ErbB2* CNG with histological and clinical characteristics such as age, neutered status, histological grade, tumor size, lymph node involvement, distant metastasis, and clinical stage in the dogs with mammary carcinoma. The presence of *ErbB2* CNG, but not ErbB2 overexpression, was significantly related to the shorter overall survival. These findings suggest that *ErbB2* CNG is a prognostic factor in dogs with mammary carcinoma.

KEY WORDS: copy number aberration, dog, ErbB2, mammary carcinoma, prognosis

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ErbB2, also known as human epidermal growth factor receptor 2 (HER2), is a cell surface receptor tyrosine kinase belonging to the ErbB2 family (ErbB1–4). Under normal circumstances, ErbB2 is involved in cell proliferation, survival, angiogenesis, and migration [12]. A normal cell has 2 copies of *ErbB2*; however, in approximately 20–40% of human breast cancer patients, the gene is present in more than two copies, known as *ErbB2* copy number gain (CNG) [4, 8, 16, 17, 22, 31]. *ErbB2* CNG is caused by gene amplification and/or polysomy and leads to ErbB2 protein overexpression [16, 22]. Patients with CNG as well as protein overexpression of ErbB2 have shown poor prognosis [4, 8, 30, 31]. Apart from being a prognostic marker, ErbB2 is also important as a therapeutic target for human breast cancer. ErbB2-targeted therapies include the use of trastuzumab and lapatinib. Trastuzumab is an anti-ErbB2 humanized monoclonal antibody. Treatment with trastuzumab significantly improves 33–52% of disease-free survival and 34–41% of overall survival in early human breast cancer patients [24, 32]. Lapatinib is a small-molecule tyrosine kinase inhibitor of ErbB2 [27, 36]. Treatment with lapatinib has prolonged progression-free survival in human breast cancer patients who had progressed on trastuzumab monotherapy [6, 7]. Given the prognostic and therapeutic implications, an assessment of ErbB2 level in a human breast cancer patient is crucial.

Canine mammary gland tumors account for approximately 50% of all tumors in female dogs [20]. Of these tumors, 41–53% are malignant [3]. Several studies have proposed canine cases as a model of human breast cancer based on many similarities that

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have been reported between the two species [1, 18, 25]. ErbB2 protein overexpression has been observed in 18–48% of canine mammary carcinoma [10, 13, 19, 26, 29], which was similar to that reported in human breast cancer (15–30%) [14]. On the other hand, data on *ErbB2* copy number in canine mammary carcinoma is limited. A previous study, using chromogenic *in situ* hybridization, has shown no *ErbB2* CNG in canine mammary carcinoma [19]. However, the sample size of the study was too small (n=6) to reach a conclusion. Another study, using digital PCR (dPCR), has shown that *ErbB2* CNG was detected in 4/20 (20%) canine mammary carcinoma [5]. To our knowledge, there is no study investigating whether *ErbB2* CNG is associated with prognosis in dogs with mammary carcinoma.

In this study, we evaluated *ErbB2* copy number in canine mammary carcinoma using a dPCR assay with formalin-fixed paraffin-embedded (FFPE) tissues and compared these data with results of ErbB2 protein expression obtained from immunohistochemistry in the same specimens. Moreover, we investigated association of *ErbB2* CNG with histological and clinical characteristics and survival in dogs with mammary carcinoma.

MATERIALS AND METHODS

Samples

A total of 59 FFPE canine mammary gland tissues (34 carcinoma, 14 adenoma, and 11 normal) were used in this study (Supplementary Table 1). Tissue samples of 11/11 normal, 8/14 adenoma, and 34/34 carcinoma were obtained from different individuals. Remaining adenoma tissues were obtained from four carcinoma cases included in this study and different udders of an adenoma case. Tissues of carcinoma and adenoma were surgically excised from clinical cases at the Veterinary Medical Center of The University of Tokyo from December 2009 to December 2015. The diagnoses were based on histopathological evaluation. Informed consent was obtained from dog owners for the use of clinical data and samples. This study did not reach the threshold for submission to a local ethical and welfare committee, since the collection and use of clinical data and samples were daily activities.

Evaluation of histological and clinical characteristics

Hematoxylin and eosin-stained specimens of the mammary carcinoma tissues were evaluated by two pathologists with Japanese College of Veterinary Pathologists board certification (J.K.C. and K.U.). Each specimen was classified and graded in accordance with a method proposed by Goldschmidt *et al* [11]. TNM classification and clinical stage at surgery were determined for each mammary carcinoma case based on medical records [21].

DNA extraction and dPCR

DNA was extracted from each tissue sample with QIAmp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. A dPCR assay established in our previous study was used for evaluating *ErbB2* copy number [28]. Primers and TaqMan MGB probes of *ErbB2* and a reference gene were designed with Primer Express Software (Thermo Fisher Scientific, Waltham, MA, USA). The reference gene was designed within a region of canine chromosome 8 (*CFA8*). The region was relatively stable in canine mammary carcinoma, according to a previous study on genome aberrations [5]. The following primers and probes were used: *ErbB2* forward, 5′-GTGGTGAGGCTGGTTTTCAGA-3′ (Position: 26104529–26104549); *ErbB2* reverse, 5′-CCTGTCCTCCCACCTCTTCAT-3′ (Position: 26104469–26104489); *ErbB2* probe, 5′-TAACCGCTAAGCAGTATGT-3′ (Position: 26104494–26104512); *CFA8* forward, 5′-TGCAGAGTTTGATTTGTTGTTTGAA-3′ (Position: 7695371–7695395); *CFA8* reverse, 5′-TGGAAGAAGGTGCATTTTTCTGA-3′ (Position: 7695315–7695337); *CFA8* probe, 5′-AATGCCTTTGACCAGTGGGTAGCC-3′ (Position: 7695347–7695370). The *ErbB2* and *CFA8* probes were labelled with 6-carboxyfluorescein (FAM) and 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC), respectively. All the primers and probes were custom made at Thermo Fisher Scientific. PCR was performed using a QuantStudio™ 3D Digital PCR system (Thermo Fisher Scientific), as described previously [15, 28]. In brief, each 15 µl reaction mixture contained 2X QuantStudio™ 3D Digital PCR Master Mix v2 (Thermo Fisher Scientific), 900 nM of each primer, 200 nM of each probe, and 20 ng of genomic DNA for FFPE canine mammary gland tissues. The PCR reaction mixtures were then loaded onto a QuantStudio™ 3D Digital PCR 20K Chip v2 (Thermo Fisher Scientific) using a QuantStudio™ 3D Digital PCR Chip Loader (Thermo Fisher Scientific). The 20K chip contained 20,000 wells, inside which DNA was randomly and uniformly distributed. Amplification was carried out using ProFlex™ 2× Flat PCR System (Thermo Fisher Scientific) under the following conditions: denaturation at 96°C for 10 min; 39 cycles at 60°C for 2 min, 98°C for 30 sec, 60°C for 2 min, after which the temperature was maintained at 10°C. Subsequently, the PCR chip was loaded on a QuantStudio™ 3D Digital PCR Instrument (Thermo Fisher Scientific). The positive and negative plots were counted, and *ErbB2:CFA8* ratios were calculated using Poisson's distribution with QuantStudio™ 3D AnalysisSuite™ version 3.1.2 (Thermo Fisher Scientific, Fig. 1).

Immunohistochemistry for ErbB2

Immunohistochemistry for ErbB2 was performed as described previously [34]. Briefly, 4 µm sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide-methanol at room temperature for 5 min and then heated in a water bath at 98°C for 40 min in target retrieval solution (pH 9.0, Dako, Glostrup, Denmark). After being cooled for an hour, the sections were washed in Tris-buffered saline (TBS) and then incubated in 8% skimmed milk-TBS at 37°C for 40 min. The sections were incubated with rabbit polyclonal anti-human c-erbB-2 oncoprotein (HER2/neu) antibody (1:100 in dilution, Dako) at 37°C for 40 min and then with Envision horseradish peroxidase-labeled anti-rabbit IgG polymer (Dako) at 37°C for an hour. The reacted

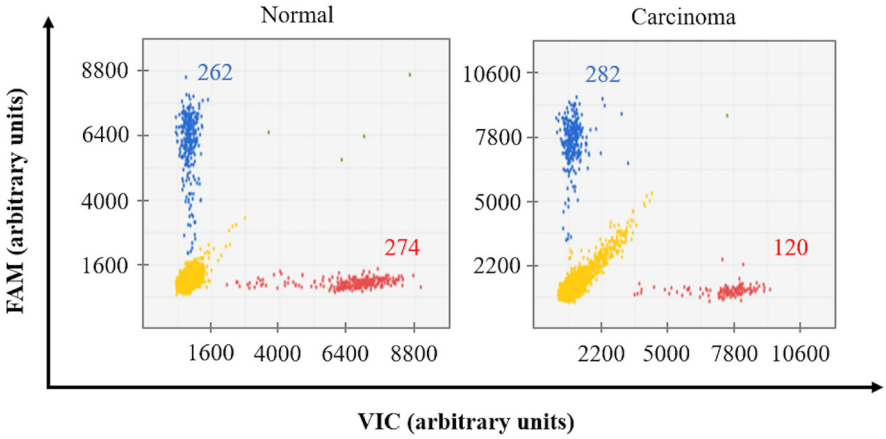

Fig. 1. Two-dimensional scatter plots of the digital PCR assay for detecting *ErbB2* copy number aberration in DNA samples obtained from a normal control dog and a dog with mammary carcinoma. *ErbB2* was labelled with 6-carboxyfluorescein (FAM), whereas the reference gene in *CFA8* was labelled with 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC). Four clusters were identified to be single-positive for FAM (blue) and VIC (red), double-positive (green), and double-negative (yellow). The ratio of blue plot points to red plot points in mammary carcinoma (282 to 120) was much higher than that in the normal control (262 to 274).

products were visualized using 3.3′-diaminobenzidine (Dojindo, Kumamoto, Japan) and 0.03% H_2O_2 in TBS. Counterstaining was conducted with Mayer's hematoxylin. Positive controls were prepared with canine urothelial carcinoma tissue (Supplementary Fig. 1A), which is known to show ErbB2 overexpression according to our previous study [34]. Negative controls were performed by omitting the primary antibody (Supplementary Fig. 1B).

ErbB2 immunoreactivities were quantified based on a guideline proposed in a previous report on canine mammary carcinoma [23]. Interpretation criteria was as follows; a score of 0 denotes no reactivity at all (Fig. 2A), a score of 1+ represents weak and incomplete membrane immunoreactivity in any percentage of cells (Fig. 2B), a score of 2+ represents strong and complete membrane immunoreactivity (chicken-wire pattern) in ≤30% of cells, or weak or moderate heterogeneous complete membrane immunoreactivity in at least 10% of cells (Fig. 2C), and a score of 3+ represents strong, complete, and homogeneous membrane immunoreactivity (chicken-wire pattern) in >30% of cells (Fig. 2D). Samples with scores of 0, 1+, and 2+ were classified as ErbB2 overexpression-negative, and those with a score of 3+ as ErbB2 overexpression-positive. Although cytoplasmic immunoreactivity was detected in some tumor cells and normal mammary gland epithelial cells, these were not taken into account.

Follow up study

For survival analyses, information regarding the current status (alive, dead, or lost) till the end of the study (April 7, 2017) was obtained for each mammary carcinoma case based on the medical records or a fax interview with the referring veterinarians. Overall survival (OS) was defined as the interval between surgery and the established cause of death of the animal at the end of the study.

Statistical analyses

To determine differences in *ErbB2:CFA8* ratio among three groups, the Kruskal-Wallis test and post hoc, Dunn's multiple comparison test were performed. Fisher's exact test was used to determine association of CNG and protein overexpression of ErbB2 with histological and clinical characteristics in canine mammary carcinoma cases. Survival curves were generated using the Kaplan-Meier method, and they were compared using the log-rank test. These statistical analyses were performed using Prism software, version 8.1.1 (Graph Pad Software, San Diego, CA, USA). $P<0.05$ was considered to be statistically significant.

RESULTS

ErbB2 copy number in canine mammary gland tissues

The median *ErbB2:CFA8* ratios (range) in the normal controls, mammary adenoma, and mammary carcinoma were 0.88 (0.69−0.98), 1.00 (0.61−1.18), and 1.10 (0.61−2.35), respectively (Fig. 3). The ratio of *ErbB2:CFA8* in the mammary carcinoma cases was significantly higher than the normal controls ($P=0.0068$) but was moderately higher than the mammary adenoma cases (Fig. 3). There was no significant difference in ratio between the normal control and the mammary adenoma cases. To determine a universal threshold of *ErbB2* CNG in mammary gland tissues, the mean + 3 standard deviation of the *ErbB2:CFA8* ratio in the normal controls was calculated and was found to be 1.12. Thus, *ErbB2:CFA8* ratio >1.12 was defined as the universal threshold. Based on the threshold value, *ErbB2* CNG was detected in 14/34 (41%) mammary carcinoma and 2/14 (14%) mammary adenoma cases (Table 1). No *ErbB2* CNG was detected in the normal controls.

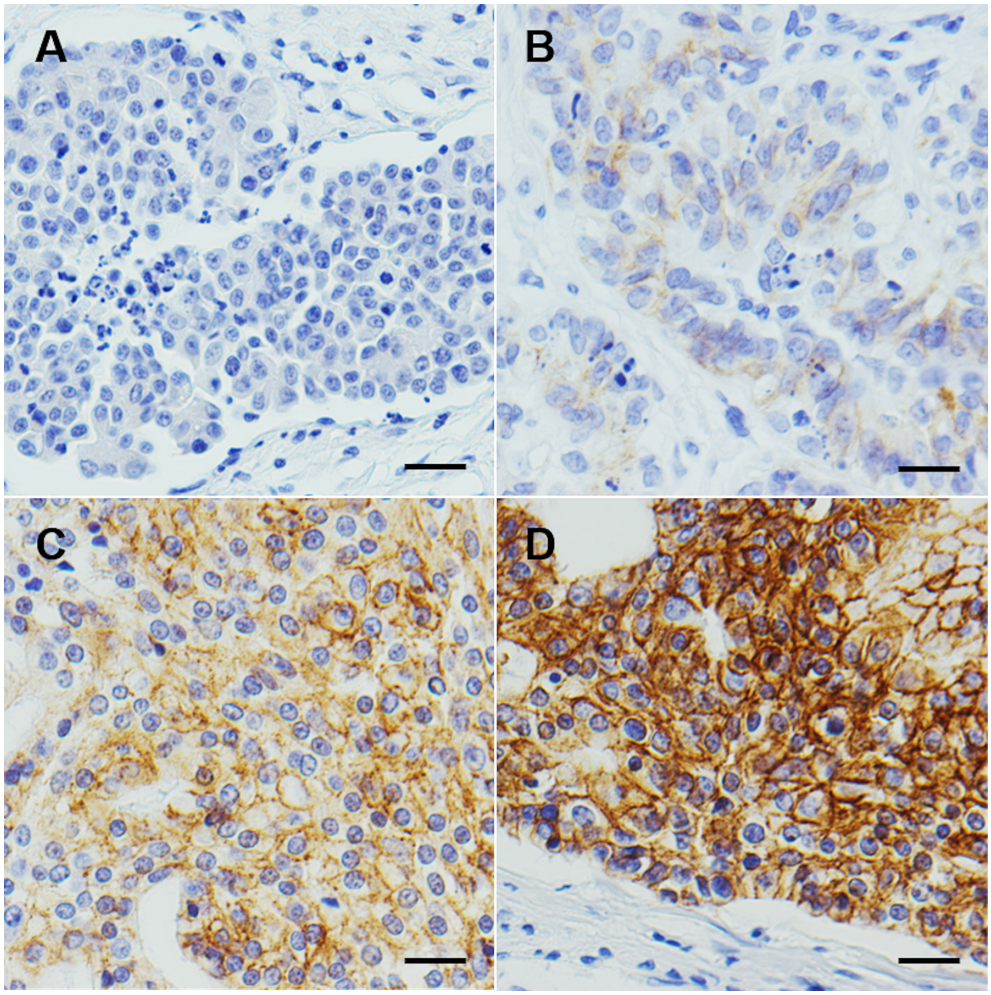

Fig. 2. Immunohistochemistry for ErbB2 in canine mammary gland tissues. No immunoreactivity in the neoplastic cells was scored as 0 (A). Weak and incomplete membrane immunoreactivity in the neoplastic cells was scored as 1+ (B). Strong and complete membrane immunoreactivity in ≤30% of the neoplastic cells was scored as 2+ (C). Strong, complete, and homogeneous membrane immunoreactivity (chicken-wire pattern) in >30% of the neoplastic cells was scored as 3+ (D). Counter-stained with Mayer's haematoxylin. Bars=25 µm.

ErbB2 protein expression in canine mammary gland tissues

ErbB2 immunoreactivity in each sample is listed in Supplementary Table 1. In the normal controls, 7/8 (88%) samples were scored as 0, and the remaining sample (13%) was scored as 1+. In the mammary adenoma, 2/14 (14%) samples were scored as 0, 10/14 (71%) were scored as 1+, and 2/14 (14%) were scored as 2+. All samples of the normal controls and mammary adenoma were classified as ErbB2 protein overexpression-negative. In the mammary carcinoma, 13/34 (38%) samples were scored as 0, 12/34 (35%) were scored as 1+, 6/34 (18%) were scored as 2+, and 3/34 (9%) were scored as 3+. ErbB2 protein overexpression was observed in 3/34 (9%) of mammary carcinomas.

ErbB2 CNG was detected in 2/3 (67%) mammary carcinoma cases with ErbB2 protein overexpression. On the other hand, ErbB2 overexpression was observed in 2/14 (14%) mammary carcinoma cases with *ErbB2* CNG. There was no significant association between CNG and protein overexpression of ErbB2 (Table 2).

Association of ErbB2 CNG and protein overexpression with histological and clinical characteristics in dogs with mammary carcinoma

There was no significant association of the *ErbB2* CNG with histological and clinical characteristics, such as histological grade, tumor size, lymph node involvement, distant metastasis, and clinical stage in the mammary carcinoma cases (Table 3). However, the *ErbB2* CNG tended to be associated with large tumor size (P=0.1157) and lymph node involvement (P=0.1345). Distant metastasis was observed only in the cases with *ErbB2* CNG. There was no significant association of the ErbB2 protein overexpression with histological and clinical characteristics in the mammary carcinoma cases (Table 3).

Impact of ErbB2 CNG and protein overexpression on survival in dogs with mammary carcinoma

All of the mammary carcinoma cases were used for survival analyses. These included 14 cases with *ErbB2* CNG and 20 without

Table 1. Copy number gain and protein overexpression of ErbB2 in canine mammary gland tissues

Sample	*n*	Copy number gain		Protein overexpression	
		Positive	Negative	Positive	Negative
Normal	11	0 (0%)	11 (100%)	0 (0%)	11 (100%)
Adenoma	14	2 (14%)	12 (86%)	0 (0%)	14 (100%)
Carcinoma	34	14 (41%)	20 (59%)	3 (9%)	31 (91%)

Data are presented as the number of cases involved in the study.

Table 2. Relationship between copy number gain and protein overexpression of ErbB2 in dogs with mammary carcinoma

	Protein overexpression		*P*
	Positive (*n*=3)	Negative (*n*=31)	
Copy number gain			
Positive (*n*=14)	2	12	0.5555
Negative (*n*=20)	1	19	

Data are presented as the number of cases involved in the study.

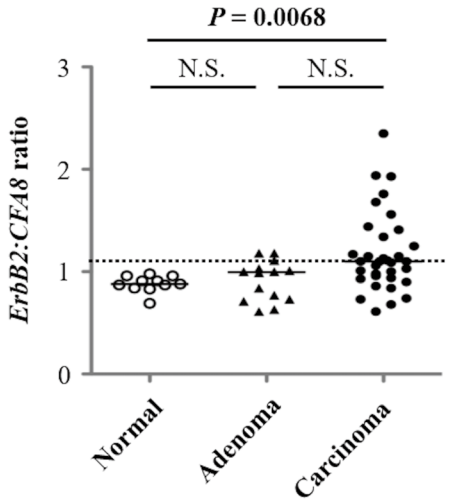

Fig. 3. *ErbB2:CFA8* ratios in canine mammary gland tissues of normal (*n*=11), adenoma (*n*=14), and carcinoma (*n*=34). Horizontal lines indicate the medians. The dotted line indicates a universal threshold (>1.12) for detecting *ErbB2* copy number gain. N.S., not significant.

Table 3. Association of copy number gain and protein overexpression of ErbB2 with histological and clinical characteristics in dogs with mammary carcinoma

	Copy number gain		*P*	Protein overexpression		*P*
	Positive	Negative		Positive	Negative	
Age						
≤Median	5	13	0.1625	1	17	0.5909
>Median	9	7		2	14	
Neutered status						
Intact	9	12	1.0000	2	19	1.0000
Spayed	5	8		1	12	
Histological grade						
I, II	12	16	1.0000	2	26	0.4525
III	2	4		1	5	
Tumor size						
T1, T2	8	17	0.1157	1	24	0.1644
T3	6	3		2	7	
Lymph node involvement						
N0	7	16	0.1345	1	22	0.2390
N+	7	4		2	9	
Distant metastasis						
M0	12	20	0.1622	2	30	0.1711
M+	2	0		1	1	
Clinical stage						
I, II, III	8	15	0.4575	1	22	0.2390
IV, V	6	5		2	9	
Postoperative chemotherapy						
Yes	5	6	1.0000	2	9	0.2390
No	9	14		1	22	

Data are presented as the number of cases involved in the study.

ErbB2 CNG. There was no significant difference in age and neutered status between the two groups (Table 3). During the study, 21/34 mammary carcinoma cases died. The dead dogs included 10 cases with *ErbB2* CNG and 11 without *ErbB2* CNG. The median OS in the cases with and without *ErbB2* CNG was 243 days (range, 79−711 days) and 515 days (range, 11−2,008 days), respectively. The OS in the cases with *ErbB2* CNG was significantly shorter than that in the cases without *ErbB2* CNG (*P*=0.0276; Fig. 4A).

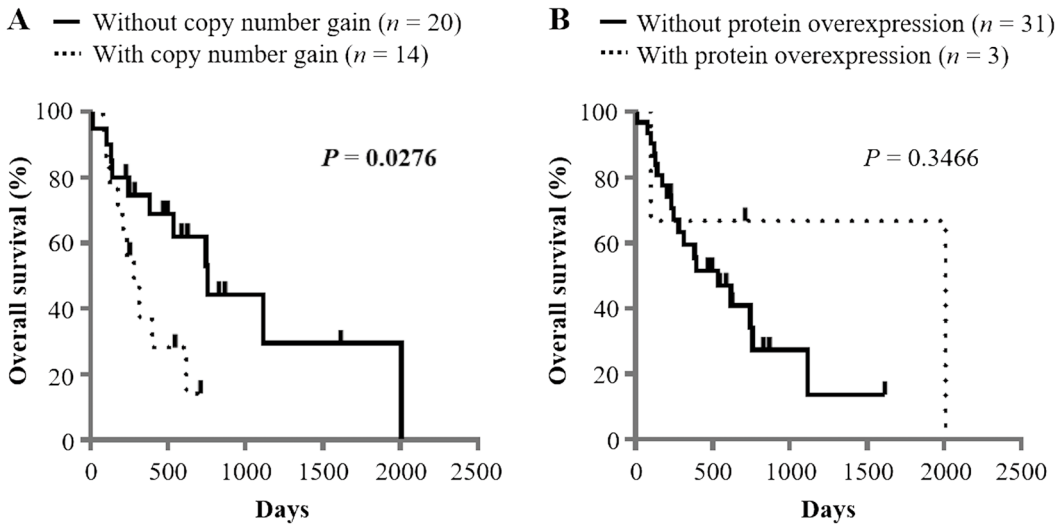

Fig. 4. Kaplan-Meier curves of overall survival in canine mammary carcinoma cases based on copy number gain (A) and protein overexpression (B) of ErbB2. Black symbols indicate censored cases.

Three cases with ErbB2 protein overexpression and 31 without ErbB2 protein overexpression were included in survival analysis. There was no significant difference in age and neutered status between the two groups (Table 3). During the study, 2 cases with ErbB2 protein overexpression and 19 without ErbB2 protein overexpression died. The median OS in the cases with and without ErbB2 protein overexpression was 711 days (range, 99−2,008 days) and 313 days (range, 11−1,615 days), respectively. There was no significant difference in the OS between the two groups (P=0.3466; Fig. 4B).

DISCUSSION

In this study, we found that *ErbB2:CFA8* ratios were significantly higher in the canine mammary carcinoma cases than in the normal controls. Based on our established threshold, *ErbB2* CNG was detected in 41% of the mammary carcinoma cases. However, the prevalence of *ErbB2* CNG was higher than that of ErbB2 protein overexpression (9%) in the same mammary carcinoma cases. There was no evidence of ErbB2 protein overexpression in 86% of the mammary carcinoma cases with *ErbB2* CNG, suggesting that just because there is detection of *ErbB2* CNG, doesn't mean this will translate to protein overexpression. Analysis of *ErbB2* mRNA expression in canine mammary carcinoma might help to explain the discrepancy between CNG and protein overexpression results. Unfortunately, however, mRNA samples of the canine mammary carcinoma were not available due to the retrospective design of the study. On the other hand, *ErbB2* CNG was detected in 67% of the mammary carcinoma cases with ErbB2 protein overexpression. This finding suggests that ErbB2 protein overexpression in canine mammary carcinoma may be due to not only CNG but also other mechanisms such as transcriptional or post-transcriptional mechanisms. In fact, it is known that ErbB2 protein overexpression is caused by increased transcription through high levels of transcriptional activators, such as action protein 2 and Yin Yang 1 in human breast cancer [2]. Further studies are needed to investigate detailed mechanisms of ErbB2 protein overexpression in canine mammary carcinoma.

The proportion of *ErbB2* CNG in canine mammary carcinoma were different between this study (41%) and previous studies (0% and 20%) [5, 19]. This may be due to differences in threshold-setting methods and assay systems. In addition, this study showed ErbB2 protein overexpression in 9% of canine mammary carcinoma, however, the proportion was lower than that in previous studies (18–48%) [10, 13, 19, 26, 29]. This discrepancy is, in part, related to variations in evaluation systems. For example, a score of 3+ was considered protein overexpression in this study, whereas scores of 2+ and 3+ were considered protein overexpression in the previous studies [10, 13]. The difference in methods between studies hamper any definitive conclusions on the ErbB2 expression in canine mammary carcinoma. To standardize the ErbB2 immunohistochemistry in canine mammary carcinoma, Pena *et al.* (2014) have proposed a guideline [23], following ErbB2 testing in human breast cancer [35]. Therefore, we used the recommended guideline.

Human breast cancer patients with CNG as well as protein overexpression of ErbB2 have shown poor prognosis [4, 8, 30, 31]. As in humans, the presence of *ErbB2* CNG in the canine mammary carcinoma cases was significantly associated with the shorter OS, suggesting that *ErbB2* CNG is involved in tumor progression. On the other hand, there was no significant association of ErbB2 protein overexpression with the OS in the canine mammary carcinoma cases. Although this may have been due to the low number of cases with ErbB2 protein overexpression, *ErbB2* CNG in canine mammary carcinoma may be a more useful prognosis indicator than ErbB2 protein overexpression.

In human breast cancer, CNG and/or protein overexpression of ErbB2 are important predictors for response to ErbB2-targeted therapies, such as those using trastuzumab and lapatinib [6, 7, 24, 32]. In this study, we detected CNG and/or protein

overexpression of ErbB2 in about half of the canine mammary carcinoma cases. Although these abnormalities were not strongly inter-related, there is considerable potential of ErbB2-targeted therapies for canine mammary carcinoma. However, there is no report on clinical trial of ErbB2-targeted therapies for dogs with mammary carcinoma at present. Further studies will be necessary to investigate antitumor effect of ErbB2-targeted therapies on canine mammary carcinoma and predict response to the therapies.

ErbB2 CNG was also detected in 14% of the canine mammary adenoma cases. However, no ErbB2 protein overexpression was observed in all of the cases. CNG and protein overexpression of ErbB2 in canine mammary adenoma are controversial. Although Martin *et al.* (2003) have reported that no *ErbB2* CNG was detected in two canine mammary adenoma cases [19], the sample size was too small. A previous study has reported no ErbB2 protein overexpression in 22 canine benign mammary tumor cases [9], whereas another study has shown that ErbB2 protein overexpression was observed in 16/32 (50%) canine benign mammary tumor cases [26]. In human benign breast diseases, *ErbB2* CNG has been detected in 6.8% of patients, and was associated with an increased risk of breast cancer [33]. Thus, the *ErbB2* CNG detected in the canine mammary adenoma cases might be associated with the risk of developing carcinoma. Further studies are necessary to investigate the association between *ErbB2* CNG and an increased risk of carcinogenesis in canine mammary adenoma.

There were some limitations to this study. First, the sample size in this study was relatively small. ErbB2 overexpression was detected only in 3 dogs. More extensive study will be needed. Second, the median percentages of tumor cells in the adenoma and carcinoma tissues were 30% (range, 10 − 90%) and 50% (range, 10 − 90%), respectively (Supplementary Table 1). A large amount of non-tumor cells may have influenced the results of the dPCR assay. Third, histological and clinical characteristics in the mammary carcinoma cases were not unified. There was no significant difference in these characteristics between the cases with *ErbB2* CNG and without *ErbB2* CNG (Table 3). However, postoperative chemotherapy may have influenced the OS in each case. Application of the dPCR assay to a larger case series with a more standardized post-diagnostic care would help to formalize the apparent association.

The present study suggested that *ErbB2* CNG is associated with adverse outcomes in dogs with mammary carcinoma. This finding provides new insights into the molecular pathogenesis of canine mammary carcinoma.

CONFLICT OF INTEREST. The authors have nothing to disclose.

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