FULL PAPER *Pathology*

Expression and significance of CHIP in canine mammary gland tumors

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ABSTRACT. CHIP (Carboxy terminus of Hsc70 Interacting Protein) is an E3 ubiquitin ligase that can induce ubiquitination and degradation of several oncogenic proteins. The expression of CHIP is frequently lower in human breast cancer than in normal breast tissue. However, the expression and role of CHIP in the canine mammary gland tumor (CMGT) remain unclear. We investigated the potential correlation between CHIP expression and mammary gland tumor prognosis in female dogs. CHIP expression was measured in 54 dogs by immunohistochemistry and real-time RT-PCR. CHIP protein expression was significantly correlated with the histopathological diagnosis, outcome of disease and tumor classification. The transcriptional level of CHIP was significantly higher in normal tissues (P=0.001) and benign tumors (P=0.009) than it in malignant tumors. CHIP protein expression was significantly correlated with the transcriptional level of *CHIP* (P=0.0102). The log-rank test survival curves indicated that patients with low expression of CHIP had shorter overall periods of survival than those with higher CHIP protein expression (P=0.050). Our data suggest that CHIP may play an important role in the formation and development of CMGTs and serve as a valuable prognostic marker and potential target for genetic therapy.
KEY WORDS: canine mammary gland tumor, CHIP, immunohistochemistry, prognosis, RT-PCR

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Mammary gland tumors are the most common tumors in female dogs and women [3, 31, 33]. Approximately half of all canine mammary gland tumors are malignant [5, 6], with a high rate of recurrence following surgical excision [28, 30]. It is crucial to find appropriate biomarkers to define the cancer risks, contribute to tumor detection and diagnosis, predict outcomes of the disease and assist in surveillance for disease recurrence. So far, many biomarkers, such as mutant *p53* and PTEN, of tumorigenesis have been found for canine mammary gland tumors (CMGTs) [22, 31]. Recently, the CHIP (Carboxy terminus of Hsc70 interacting protein) gene has come to be thought of as a tumor suppressor gene with prognostic significance. When examined in humans, CHIP expression has also been reported to be decreased in human mammary and gastric cancer [29, 37].

CHIP, which is encoded by the *STUB1* gene, is an E3 ubiquitin ligase that induces ubiquitination [7, 10] and degradation of several oncogenic proteins, including mutant P53 [25, 36], estrogen receptor A [11], c-ErbB2/neu [40], Dbl [19], Smad3 [39], hypoxia inducible factor 1a [4], Runx1 [34], Met receptor [16] and SRC-3 [18]. It could also act as a suppressor of tumor metastasis. CHIP possesses a tetratricopeptide repeat (TPR) domain, which interacts with the molecular chaperones Hsc/Hsp70 and Hsp90, and a carboxyl-terminal U-box domain with E3 ubiquitin ligase activity, which functions as a link between the chaperone and proteasome systems [2]. In humans, there is substantial evidence showing that CHIP functions as a tumor suppressor. Some recent studies indicate that the abundance of CHIP inhibits metastatic potential, and knockdown of CHIP increased the microvessel density in human breast and gastric cancers [15, 18, 29, 37]. However, the function and prognostic role of CHIP expression in CMGTs have not been well studied. The aim of our study was to assess CHIP expression and its possible use as a prognostic marker in CMGTs.

MATERIALS AND METHODS

Animal tissue and histological classification: All of the mammary gland tumor specimens including five normal mammary glands were collected from the Veterinary Teaching Hospital of China Agricultural University between July 2009 and September 2011. Mammary gland tumors were surgically removed from 49 female dogs of different breeds aged between 2 and 17 years old (mean=10 years old). Normal mammary tissues were obtained from five healthy experimental dogs, and the procedures were approved by the Animal Welfare Committee of the Department of Clinical Veterinary Medicine of China Agricultural University. Two portions of each mammary gland were collected from each dog. Samples used in RT-PCR assay were frozen immediately in liquid nitrogen after surgical removal. Samples for immunohistochemistry (IHC) were fixed in 10% neutral buffered formalin and were embedded in paraffin wax by standard histological methods. Tissue blocks were sectioned at 3 μm and stained with hematoxylin and eosin (HE). Serial 3 μm sections were used for IHC. Each section was evaluated by three independent pathologists blinded to each other.

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The histological type was assessed based on classification and grading of canine mammary gland tumors in 2011 [14]. Histological grading of mammary carcinomas was assessed according to a previously described method of classification [9, 20]. The canine mammary gland carcinomas were classified as simple, solid, complex, spindle cell or sarcoma.

Overall survival time was the period between surgery and death due to the malignant tumor. Dogs dying of non-tumor-related causes were removed from the study. Follow-up data were obtained by consulting the medical records in the hospital and by telephone contact with the owners of the animals.

Immunohistochemistry staining: Three-micrometer-thick sections were first dewaxed in xylene and rehydrated in graded alcohols. The slides were immersed in 3% hydrogen peroxidase for 20 min to quench endogenous peroxidase activity. They were then placed into jars containing citric acid buffer to unveil the antigen, and the retrieval was performed in a microwave oven at 98°C for 20 min. After the jars were cooled to room temperature at 25°C, the slides were covered with 10% goat serum in PBS for 30 min at room temperature. After blocking nonspecific binding, the slides were incubated with the primary antibody overnight at 4°C in a moist chamber. Rabbit polyclonal anti-CHIP (Anti-STUB1 polyclonal antibody, Abcam, Cambridge, U.K.) used as the primary antibody was diluted 1:200 with PBS. After being thoroughly rinsed three times in PBST for 10 min each, the slides were incubated with the secondary antibody (HRP-Labeled anti-rabbit antibody, Santa Crus Biotechnology, Dallas, TX, U.S.A.) according to the manufacturer's instructions. The slides were thoroughly washed 3 times again, and then, the color was developed with 3, 3′-diaminobenzidine tetrahydrochloride (DAB kit, ZSGB-BIO, Beijing, P.R. China) for 10 min. The sections were counterstained with hematoxylin, dehydrated with graded alcohol and xylene, and mounted with a cover slip. Negative controls were obtained by replacing the primary antibody with normal rabbit serum.

Assessment of immunohistochemistry: CHIP expression was evaluated independently by three pathologists blinded to the clinical data. A semiquantitative immunoreactivity score was applied in this text, as reported elsewhere [38, 41]. The intensity of immunostaining was scored on a scale of 0–3 (0, negative immunostaining; 1, weak immunostaining; 2, moderate immunostaining; and 3, strong immunostaining). The percentage of immunoreactive cells was scored as 1 (0–25%), 2 (26–50%), 3 (51–75%) or 4 (76–100%). Multiplication of both resulted in an immunoreactive score (IRS) ranging from 0 to 12 for each tumor. Additionally, specimens with an IRS ≤ 4 and those with an IRS>4 were classified as having low and high expression of CHIP protein, respectively [38, 41]. For accurate analysis, the number of immune-labeled cells was assessed based only on the number of positive cells among the neoplastic cells within 20 selected fields.

RNA isolation and cDNA synthesis: RNA isolation was performed with the use of RNAiso Plus (Takara; Dalian, Liaoning, P.R. China) according to the manufacturer's instructions (Takara; code No. 9108/9109). Approximately 1 μg of total RNA was reversely transcribed to cDNA using avian myeloblastosis virus (AMV) reverse transcriptase and oligo (dT) primers (Takara).

Real-time RT-PCR: The primers for real-time RT-PCR were designed using the Primer 5.0 software. The primers were 5′ CCT ACC TCA CTC GGC TTA TTG T 3′ (forward) and 5′ TCG TCC ACC TGG GAG AAA A 3′ (reverse) for *CHIP* and 5′-ATA TCG CTG CGC TTG TGG TC −3′ (forward) and 5′- CCG TGC TCA ATG GGG TAC TTC-3′ (reverse) for *β-actin*; *β-actin* mRNA for each sample was used as an internal control, and the Ct value was normalized to *β-actin* mRNA for each sample.

The transcriptional level of CHIP was determined in triplicate by real-time RT-PCR using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Briefly, the reaction mixture contained 2 μl of cDNA template, 10 μl of DNA SYBR Green qPCR mix (Takara) and 1 μl of each primer. The RT-PCR protocol was as follows: initial denaturation at 95°C for 30 sec; denaturation at 95°C for 10 sec,annealing at 60°C for 30 sec, extension at 72°C for 30 sec and fluorescent data acquisition at 72°C for 1 min (36 cycles), and final extension at 72°C for 5 min to form full duplex DNA. The specificity of the amplified products was checked by a melting curve analysis following the completion of PCR. The melting curve protocol used was heating from 60°C to 95°C at a rate of 0.3°C for 1 min per step.

Statistical analysis: Statistical analysis of the data was performed using the GraphPad Prism 5.0 or IBM SPSS statistics 20 computer software. Statistically significant variations of noncontiguous variables between different groups were determined using the chi-square test. The survival curve was analyzed using the log-rank test method. Multiple comparisons of continuous variables were analyzed using the LSD method. $P<0.05$ was considered to indicate a statistical difference, and $P<0.01$ was considered to indicate a significant difference.

RESULTS

Expression of CHIP protein in canine mammary gland tissues by immunohistochemistry: The histological types of 54 canine mammary gland tissues and their CHIP expression levels are summarized in Table 1. According to the canine mammary tumor classification in 2011 [14], 41 of the 54 cases were canine malignant mammary gland tumors, belonging to the following histopathology types: simple carcinoma (29.2%), solid carcinoma (46.3%), complex carcinoma (12%), spindle cell carcinoma (7.3%) and sarcoma (5.2%). Benign tumors were confirmed in 8 cases. There were also 5 normal mammary tissues. Immunohistochemical staining of CHIP protein in canine mammary gland tissues (Fig. 1) showed that expression of CHIP protein was mainly localized in the cytoplasm but was occasionally present in the nucleus. We also found that CHIP expression in myoepithelial cells was low in our samples. CHIP protein is abundant in normal mammary tissue, and the cell type showing a positive reaction was the luminal epithelial cell (Fig. 1A). The expression of CHIP could be detected in most of the benign tumors (Fig. 1B) and in a low percentage of carcinomas

Table1. Histopathological diagnosis, immunohistochemistry of CHIP, outcome of disease, overall survival time and grade for the 54 dogs

Sample	HD	IHC	Outcome	OS	Grade
1	Carcinosarcoma	Low	Death-metastasis	<6 months	II
2	Fibrosarcoma	Low	Death	>18 months	II
3	Complex carcinoma	Low	Death-metastasis	<6 months	II
4	Spindle cell carcinoma	Low	Alive-recurrence	>18 months	II
5	Solid carcinoma	Low	Death-recurrence	6 to 18 months	III
6	Solid carcinoma	Low	Alive	>18 months	II
7	Spindle cell carcinoma	Low	Alive	>18 months	II
8	Solid carcinoma	Low	Death	6 to 18 months	III
9	Solid carcinoma	Low	Death-metastasis	<6 months	III
10	Complex carcinoma	Low	Alive	>18 months	II
11	Tubulopapillary carcinoma	Low	Death-metastasis	<6 months	III
12	Spindle cell carcinoma	Low	Alive	>18 months	II
13	Solid carcinoma	Low	Death-recurrence	>18 months	II
14	Tubulopapillary carcinoma	Low	Death	6 to 18 months	II
15	Tubulopapillary carcinoma	Low	Death	>18 months	II
16	Tubulopapillary carcinoma	Low	Alive	>18 months	I
17	Solid carcinoma	Low	Death-metastasis	<6 months	III
18	Solid carcinoma	Low	Alive	>18 months	II
19	Solid carcinoma	Low	Death-recurrence	<6 months	II
20	Solid carcinoma	Low	Death-metastasis	<6 months	III
21	Tubulopapillary carcinoma	Low	Alive	>18 months	I
22	Solid carcinoma	Low	Death	>18months	III
23	Solid carcinoma	Low	Alive	>18 months	II
24	Tubulopapillary carcinoma	Low	Alive	>18 months	II
25	Tubulopapillary carcinoma	Low	Alive	>18months	II
26	Tubulopapillary carcinoma	Low	Alive	>18months	I
27	Solid carcinoma	Low	Death-metastasis	<6 months	III
28	Solid carcinoma	Low	Death	>18 months	I
29	Benign	Low	Alive	>18 months	
30	Complex carcinoma	High	Alive	>18 months	I
31	Solid carcinoma	High	Alive	>18 months	III
32	Tubulopapillary carcinoma	High	Alive	>18 months	II
33	Complex carcinoma	High	Alive	>18months	II
34	Tubulopapillary carcinoma	High	Alive	>18 months	II
35	Solid carcinoma	High	Death-euthanasia	<6 months	III
36	Solid carcinoma	High	Death	6 to 18 months	II
37	Tubulopapillary carcinoma	High	Alive	>18 months	I
38	Solid carcinoma	High	Alive	>18months	I
39	Solid carcinoma	High	Alive	>18 months	III
40	Solid carcinoma	High	Alive	>18 months	II
41	Tubulopapillary carcinoma	High	Alive	>18 months	I
42	Complex carcinoma	High	Death-metastasis	6 to 18 months	II
43	Benign	High	Alive	>18 months	
44	Benign	High	Alive	>18 months	
45	Benign	High	Alive	>18 months	
46	Benign	High	Alive	>18 months	
47	Benign	High	Alive	>18 months	
48	Benign	High	Alive	>18 months	
49	Benign	High	Alive	>18 month	
50	Normal	High			
51	Normal	High			
52	Normal	High			
53	Normal	High			
54	Normal	High			

HD: Histopathological diagnosis, IHC: Immunohistochemistry, OS: Overall survival (the period between surgery and death due to malignant tumor).

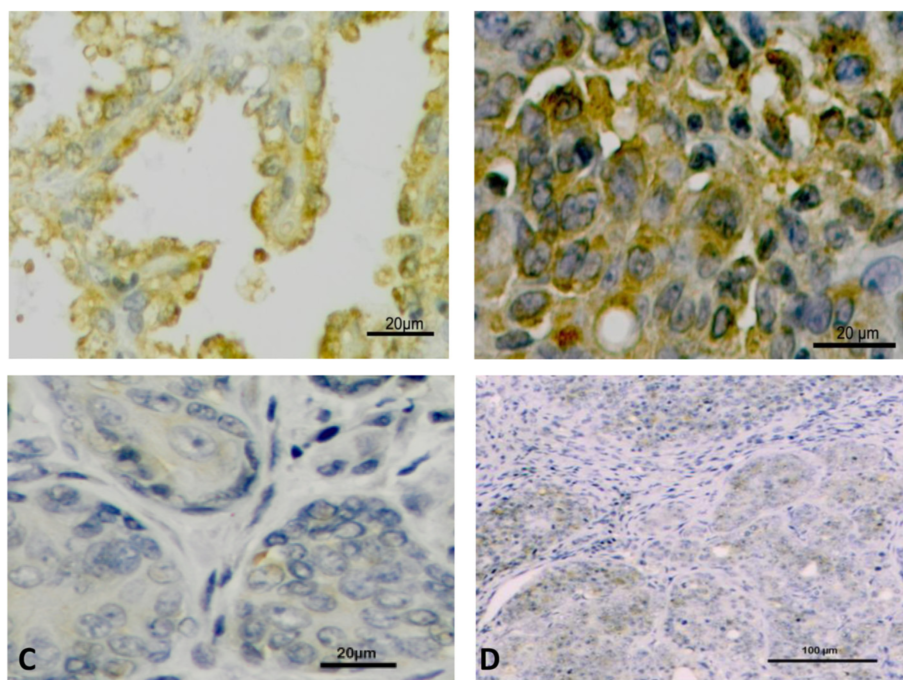

Fig. 1. Sample of immunohistochemical staining of CHIP in CMGT and normal mammary tissue. (A) Normal mammary tissue with an abundance of CHIP protein, (B) Benign tumor with an abundance of CHIP protein, (C) Malignant tumor with low expression of CHIP protein, (D) Complex carcinoma with low expression of CHIP protein.

Table 2. Correlation between histopathological diagnosis and CHIP expression in 49 canine mammary gland tumors

Histopathological diagnosis	IHC		*P*
	High	Low	
Simple	4	8	
Solid	6	13	
Complex	3	2	
Spindle	0	3	
Sarcoma	0	2	0.030*
Benign	7	1	
All	20	29	

*χ^2 test, *P* value.

Table 3. Correlation between immunostaining and outcome of disease

Immunostaining	Outcome		*P*
	Death	Alive	
High	3	10	
low	16	12	0.034*
All	19	22	

χ^2 test, *P* value.

(Fig. 1C and 1D). The CHIP protein level was low in 68% of the malignant tumors (28/41) and high in 87% of the benign tumors (7/8). We also found that the CHIP protein level was low in myoepithelial components (5/5).

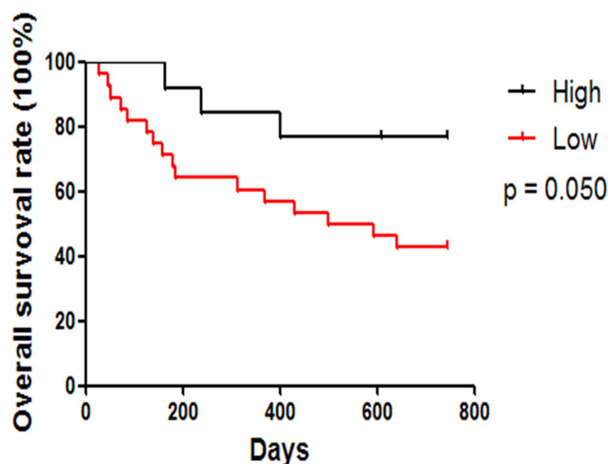

Fig. 3. Log-rank test curves for malignant tumors with high CHIP expression (IHC score >4) and low CHIP expression (IHC ≤4). Dogs with high CHIP expression had longer survival times than those with low CHIP expression (*P*=0.050).

Correlations between CHIP protein expression levels and clinicopathological variables: The immunohistochemical tests of CHIP demonstrated a significant correlation between the CHIP expression and histopathological diagnosis (*P*=0.007, Table 2). As described in Table 3, the relationship between outcome and immunostaining was statistically different (*P*=0.034). The comparative histopathological diagnosis

Table 4. Correlation between histopathological diagnosis and overall survival time for 41 dogs having malignant mammary gland tumors

Histopathological diagnosis	Overall survival time in months			P
	<6	6–18	>18	
Simple	1	1	10	
Solid	6	3	8	
Complex	1	1	3	
Spindle	0	0	3	0.531*
Sarcoma	1	0	1	
All	9	5	25	

*χ^2 test, *P* value.

Table 5. Correlation between IMC and histological grading

Histological grading	IHC		P
	High	Low	
I	4	4	
II	6	16	
III	3	8	0.4639*
All	13	28	

*χ^2 test, *P* value.

and overall survival time did not demonstrate a statistical concordance (Table 4). There were also no correlations between CHIP immunostaining and histological grading (Table 5).

Transcriptional level of CHIP in canine mammary gland tissues by RT-PCR: The transcriptional level of *CHIP* in normal mammary gland tissues differed significantly from those in malignant tumors (*P*=0.001), however, no differences were detected between normal and benign tumor tissues (*P*=0.284) (Fig. 2A). The transcriptional level of *CHIP* was significantly different between benign and malignant tumors (*P*=0.009) (Fig. 2A). There was also a good concordance between the *CHIP* transcription level detected by RT-PCR and CHIP protein expression examined by immunohistochemistry (*P*=0.0102) (Fig. 2B).

Association of CHIP protein expression in canine malignant mammary tumors with overall survival: The follow-up time was at least 18 months after tumor resection. Single variable survival analysis showed that CHIP expression was a significant prognostic factor for overall survival (*P*=0.050) (Fig. 3). Patients with a lower CHIP expression level had a poorer overall survival rate.

DISCUSSION

Human breast cancer and canine mammary gland carcinoma have the similar epidemiology and clinic pathology. Canine mammary gland carcinomas are the most common life-threatening disease in small animal clinic practice, which has as yet no effective clinical treatment. Therefore, it is important to discover a practical potential treatment target in canine mammary cancer.

CHIP is known to be involved in ubiquitination and degradation of certain oncoproteins, such as NF-κB, SRC-3 and mutant p53 [17, 18, 25, 32]. Previous research has shown that NF-κB is a useful prognostic factor for canine mammary gland tumor [24]. It regulates downstream genes, including *IL-6*, *IL-8*, *MMP-2*, *VEGF* and *cyclooxygenase-2*, to promote proliferation, survival, angiogenesis and metastasis of tumors [12, 21]. A previous study in humans also showed that overexpression of CHIP could suppress expression of NF-κB downstream genes, especially IL-8 [37]. Clinical studies have shown that IL-8 is upregulated in several human malignancies, including melanoma [26], colon cancer [8], non-small cell lung cancer, gastric cancer [23] and breast carcinoma [34], and is also linked to tumor angiogenesis, metastatic phenotype and overall poor prognosis [35]. SRC-3 is a steroid receptor coactivator, and SRC-3 overexpression has been detected in multiple cancers, including breast, gastric and prostate cancers [1, 13, 37]. In breast cancer, SRC-3 overexpression is associated with high levels of HER2, tamoxifen resistance and poor overall survival time [13, 27]. P53 is one of the most intensively studied tumor-suppressor proteins. It has been clarified that the mutant p53 proteins can gain new functions favoring the maintenance, insurgence, spreading and chemoresistance of malignant tumors [25, 36]. To elucidate whether or not a decrease in CHIP protein amount is associated with malignant proliferation of canine secretory epithelial neoplastic cells, a further study, e.g, analysis of CHIP degraded oncoproteins by immunostaining or western blotting, is needed.

Wang *et al*. reported that CHIP is a novel suppressor of tumor angiogenesis in human gastric cancer [37]. Studies of xenografts in nude mice indicated that gastric cancers overexpressing CHIP could reduce blood vessel formation, suggesting that CHIP may suppress angiogenesis in the tumor [37]. In addition, overexpression of CHIP also suppresses cell adhesion and invasion [37]. Also, Jan *et al.* found that reduced CHIP expression is related to unfavorable tumor grade, advanced pathological stage, larger tumor size and poor overall survival in breast cancer patients [15]. Taken together, the previous data presented here show that CHIP protein was significantly correlated with cancer progression and was an independent prognostic marker of overall survival in human cancer patients. Nevertheless, there are no studies focusing on CHIP expression and its clinical relevance in canine mammary cancer. In this study, we investigated the clinical relevance of CHIP in the canine mammary gland tumor.

Previous investigations have demonstrated a significant reduction in the transcriptional level of *CHIP* in a high percentage of human breast cancers versus normal mammary glands and benign mammary tumors and have also reported that the CHIP protein and *CHIP* gene transcription levels correlate well [18, 29]. In this article, we reported that the *CHIP* mRNA level was significantly correlated with the CHIP protein level, suggesting that the CHIP protein level is dependent on the amount of mRNA, which was consistent with the above previous studies.

So far, there are no published studies focusing on the relationship between the CHIP expression level and the histological grading, subtype and outcome in CMGTs. The

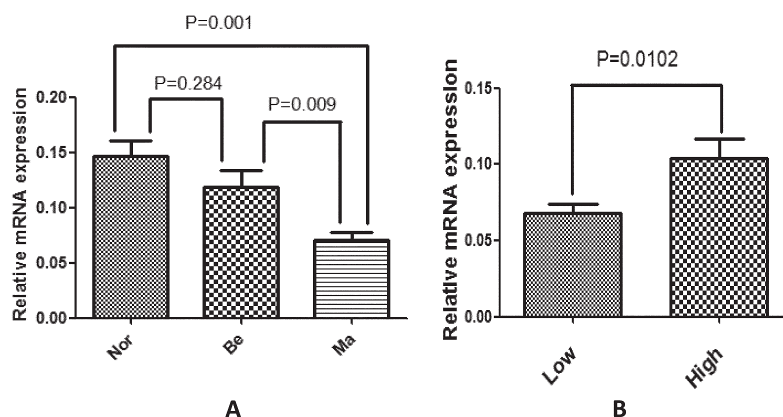

Fig. 2. Quantitative analysis of *CHIP* transcription level in normal mammary tissue, benign tumors and malignant tumors. After *CHIP* transcription levels were quantitatively analyzed by real-time RT-PCR, relative expression levels (RELs) of *CHIP* were normalized to *β-actin*. The columns and error bars represent the means and their standard errors. (A) Comparisons of RELs of *CHIP* between normal mammary gland tissues (Nor) and benign (Be) or malignant tumors (Ma) and between Be and Ma are shown in the figure. The expression levels in normal mammary tissues were significantly (P=0.012) higher than those in malignant tumors. While no significant difference was observed between normal mammary and benign tissues, a significantly (P=0.0112) higher expression was noted in the benign tumors than in the malignant tumors. (B) Comparisons of RELs of *CHIP* in mammary gland tumors with different CHIP protein expression levels. The *CHIP* gene amplification levels of tumors with high expression of CHIP protein are significantly higher than those of tumors with low expression of CHIP protein (P=0.0102).

relation between histological grading and CHIP expression in CMGTs is not with that found in human studies, but the relation between CHIP protein expression and subtype shows statistical concordance, which is consistent with human research. A shorter overall survival was observed, which was significantly associated with low CHIP expression in CMGTs in this study, and similar findings have been observed in human breast cancer.

To our knowledge, this is the first study describing CHIP protein expression analysis in CMGTs. The finding of low expression of CHIP protein in the canine mammary carcinoma and its possible role in the prognosis of this disease are clinically relevant. Moreover, agents with CHIP-enhancing activity might provide an effective strategy for treatment of breast cancer for both dogs and humans, and such agents merit further investigation.

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