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Clinicopathological analysis of expression of enhancer of zeste homologue 2 in canine mammary carcinoma

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Abstract

Introduction: Enhancer of zeste homologue 2 (EZH2) is the human homologue of *Drosophila zeste* gene enhancer. The aim of this study was to determine the expression of EZH2 in canine mammary carcinomas (CMCs) and its relationship with clinicopathological features. **Material and Methods:** The expression of EZH2 mRNA and protein in 53 CMC tissue and 8 normal mammary gland tissue samples was measured by quantitative real-time PCR and immunohistochemical staining assay, respectively. The relationship between EZH2 protein expression and clinicopathological features was analysed by $\chi 2$ test to further explore the clinical significance of EZH2 in CMCs. **Results:** Compared with normal mammary gland tissues, EZH2 mRNA expressions were significantly increased in CMC tissues ($P < 0.01$). Moreover, normal mammary glands did not express the EZH2 protein but carcinomic glands did, and expression increased in CMCs with high histological grades, especially in histological grade II ($P < 0.05$). However, EZH2 expression was not related to age, tumour size, or metastasis ($P > 0.05$). The expression of EZH2 in one type of CMC was not significantly different from the expression in any other type ($P > 0.05$). **Conclusion:** EZH2 is highly expressed in CMCs, indicating that it can be used as a molecular marker for early diagnosis, prognosis, or therapy of CMCs.

Keywords: canine mammary carcinoma, EZH2, clinicopathological examination.

Introduction

Enhancer of zeste homologue 2 (*EZH2*) is a human homologou gene of *Drosophila zeste* enhancer located on chromosome 7q35 (5). It belongs to the *PcG* gene family and is a group of key epigenetic regulators that repress transcription (10). The ability of EZH2 proteins to bind to and regulate several oncogenic and various tumour suppressor gene products suggests that they may play a role in tumourigenesis (9). The enhancer homologue is essential in the pathophysiology and development of tumours (20). Various human tumour tissues including prostate, breast, bladder, and liver cancer exhibit overexpressed EZH2 and it is also upregulated in human breast cancer (8, 2, 9, 26). The proteins's presence is closely related to tumour size, infiltrative depth, tumour stage, lymph node metastasis, and drug resistance, indicating that it acts as both a tumour oncogene and a prognostic indicator (26). Several studies have suggested that inhibition of EZH2 expression or function suppresses tumour cell proliferation, invasion, and metastasis (30, 29). EZH2 is up-regulated in gastric cancer tissues compared with adjacent normal tissues, associating with a relatively poor prognosis. However, EZH2 knockdown by small interfering RNA suppresses cell proliferation and invasion by regulating p21 expression (31). Inhibition of EZH2 is possible among other ways by utilising ZLD1039, a highly selective inhibitor suppressing breast tumour growth and metastasis in mice (25). In the inverse position, tumour malignancy development through upregulation of EZH2, and this mechanism

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indicates that EZH2 can be a biomarker and potential therapeutic target for tumour diagnosis and treatment.

Canine mammary carcinoma (CMC) is a life-threatening tumour and a worldwide major cause of cancer-related morbidity and mortality in dogs (1). The incidence of CMC gradually increases with age, and about 50% are malignant tumours (4). These carcinomas are caused by complex multi-step and multi-gene processes, including tumour suppressor gene inactivation, oncogene activation, and related key signalling protein changes (17). Presently, early diagnosis and surgical excision are the most effective treatment methods for CMC. However, it is apt to recur and metastasise. Chemotherapy is mainly used as an adjuvant modality for CMC, because of its relatively minor efficacy (27). Although several biomarkers, such as the carcinoembryonic antigen, HER-2, and microRNA, have been considered for CMC treatment, there is no universally-accepted biomarker for its clinical treatment (28, 22, 16, 24). Veterinary field studies have shown that EZH2 expression is positively associated with tumour aggressiveness; EZH2 is also known to be overexpressed in canine lymphoma, melanoma, basal cell tumors, squamous cell carcinoma, and prostate cancer (6). Although a few studies have assessed EZH2 expression in CMC, it is unclear whether EZH2 can be used as a clinical biomarker for CMC.

In the present study, quantitative real-time PCR and immunohistochemistry were used to analyse the mRNA and protein levels of EZH2 in CMC and normal mammary gland tissue samples. The relationship between EZH2 expression in CMC tissues and clinicopathological features was also investigated to analyse the role of EZH2 in tumourigenesis and the development of CMCs. It is hoped that this study provides new insights into this canine cancer and a theoretical basis for the clinical diagnosis and treatment of CMCs.

Material and Methods

Sample collection. Samples were provided by the veterinary hospital of the Northeast Agricultural University in Harbin, China. A total of 53 samples of CMC tissue formalin-fixed paraffin-embedded were studied. They included 41 invasive ductal carcinomas (IDC), 6 intraductal papillary carcinomas, 3 invasive micropapillary carcinomas, and 3 ductal carcinomas *in situ* (DCIS), which had been identified from 2015 to 2018 in 53 dogs. The patients were among 3 and 15 years of age with an average age of 8.76 ± 3.4, and the average weight of the dogs was 4.52 ± 2.63 kg. Most of them were mixed-breed dogs, and some poodles, bichon frise, pekingese, cocker spaniel, chihuahua and golden retriever. The tumours were mainly located in the anterior abdomen, posterior abdomen, inguinal side and posterior chest. In addition, eight normal canine mammary gland tissue samples were used as controls and they came from dogs between 12 and 20 months of age weighing 5–8 kg. None of the dogs underwent ovarian hysterectomy, nor did they receive preoperative chemotherapy or radiotherapy. Histopathological classification and grading of CMC samples were conducted as previously described (11).

Quantitative real-time PCR (qPCR). This method was used to determine the relative mRNA levels of EZH2 in tissue samples. Total RNA was isolated from each tissue sample using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was reverse transcribed to obtain cDNA using PrimeScript RT reagent Kit (Perfect Real Time) (TaKaRa, Kusatsu, Japan). Briefly, 1 μg of RNA of each sample, 2.0 μL of 5× gDNA Eraser Buffer, 1.0 μL of gDNA Eraser, and RNase-free ddH_2O were added to reach 10 μL, then the solution was reacted at 42°C for 2 min. A 1.0 μL volume of PrimeScript RT Enzyme Mix I, 1.0 μL of RT Primer Mix, 4.0 μL of 5 × PrimerScript Buffer, and 4.0 μL of RNase-free ddH_2O were added to the system, incubated at 37°C for 15 min, and then reacted at 85°C for 5 s.

The TB Green Premix Ex Taq II (Takara) premix was used for real-time PCR in an ABI7500 System (Applied Biosystems, Foster City, CA, USA). Primers of canine *EZH2* and *GAPDH* genes were designed using Primer 5.0 software (Premier) according to the gene sequence obtained from NCBI GenBank. The primer sequences of canine EZH2 (XM_038688969.1) were upstream F5′-CGGCACACTGCAGAAAGATA-3′R and downstream F5′-CTATCACACAAGGGC ACGAA-3′R. The primer sequences of *GAPDH* were upstream F5′-GCTGCCAAATATGACGACATCA-3′R and downstream F5′-GTAGCCCAGGATGCCTTT GAG-3′R. The q-PCR parameters were denaturation at 95°C for 5 s, annealing at 60°C for 20 s and elongation at 65°C for 15 s. The PCR reaction consisted of 40 cycles. The transcription level of the *EZH2* gene was calculated using the $2^{-\triangle\triangle CT}$ method.

Immunohistochemistry. The expression of EZH2 in CMC tissue samples was determined using an immunohistochemistry assay. The formalin-fixed paraffin-embedded tissue specimens were cut into 3-μm-thick sections. Rabbit monoclonal anti-EZH2 antibody (bsm-60001R) was obtained from Bioss (Beijing, China) and used at a dilution of 1:100. The immunostaining process involved deparaffinisation and high-pressure antigen retrieval at pH 6.0, peroxidase blocking, incubation with primary antibody at 4°C overnight, post-primary Poly-HRP-goat-anti-rabbit IgG incubation, diaminobenzidine chromogen treatment, and haematoxylin counterstain. Negative control slides were prepared by replacing primary antibodies with phosphate-buffered saline.

Evaluation of immunoreactivity. Yellow or brown-yellow granules in the nucleus indicated positive expression of EZH2. A microscope (Eclipse 50i; Nikon, Tokyo, Japan) was used to visualise the tissues

under a high-power light source (ten views per slice). The immunohistochemistry score (Histoscore) was calculated using a semi-quantitative double-blind method. The staining intensity (0, 1+, 2+, or 3+) and the proportion of cells with EZH2 were also determined (6, 7).

Statistical analysis. SPSS 22.0 software (Chicago, IL, USA) was used for all statistical analyses. Data are expressed as mean ± standard deviation (X ± SD). One-way ANOVA was used to determine statistical differences. The relationship between EZH2 expression and clinicopathological features, including the age of dogs, tumour size, metastasis state correlated with histological grade, and histological type, was analysed using the $\chi 2$ test. $P < 0.05$ and $P < 0.01$ were considered significant and extremely significant differences, respectively.

Results

Messenger RNA levels of EZH2 in CMC. The mRNA levels of EZH2 in CMC and normal canine mammary tissues were assessed using qPCR. Compared with normal tissues, the mRNA levels of EZH2 were significantly elevated in IDC and DCIS (Fig. 1), suggesting that CMCs are associated with the upregulation of EZH2.

Expression of EZH2 protein in CMC. The expression of the EZH2 protein in CMC tissue and normal tissue was detected using immunohistochemistry. It was mainly expressed in the nucleus of malignant tumour cells and a small number of mammary gland epithelial cells. Moreover, EZH2 was diffusely and uniformly distributed in the cancer nests. The protein was significantly downregulated in normal tissues (Fig. 2A). There was strong positive nuclear staining of EZH2 in infiltrating ductal carcinoma (Fig. 2B) and DCIS tissue (Fig. 2E). In contrast, there was weak positive staining in intraductal papillary carcinoma (Fig. 2C) and infiltrating micropapillary carcinoma tissue (Fig. 2D). EZH2 was significantly positively upregulated in IDC and DCIS (positive expression rates; 60.97% (25/41) and 33.33% (1/3), respectively). However, its expression was relatively weak in intraductal papillary carcinomas and invasive micropapillary carcinomas (positive expression rates: 33.33% (2/6) and 66.67% (2/3), respectively). These results indicate that EZH2 is highly expressed in CMCs.

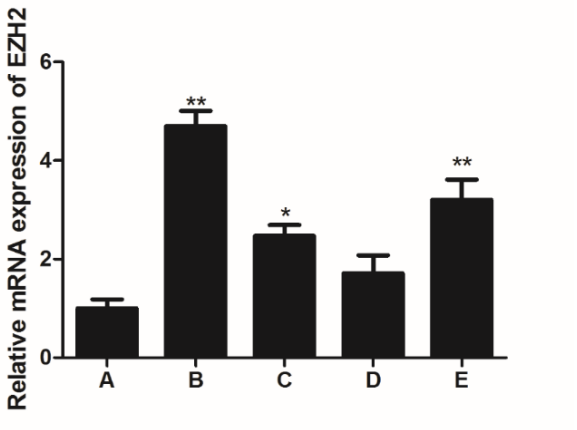

Fig. 1. Transcription level of the *EZH2* gene in canine normal mammary gland and CMC tissues
A – normal canine mammary tissues; B – infiltrating ductal carcinoma; C – intraductal papillary carcinomas; D – infiltrating micropapillary carcinoma; E – ductal carcinoma *in situ*
Results are presented as mean ± SD of tissues in the group. * $P < 0.05$ and ** $P < 0.01$ show significantly different by one ANOVA test

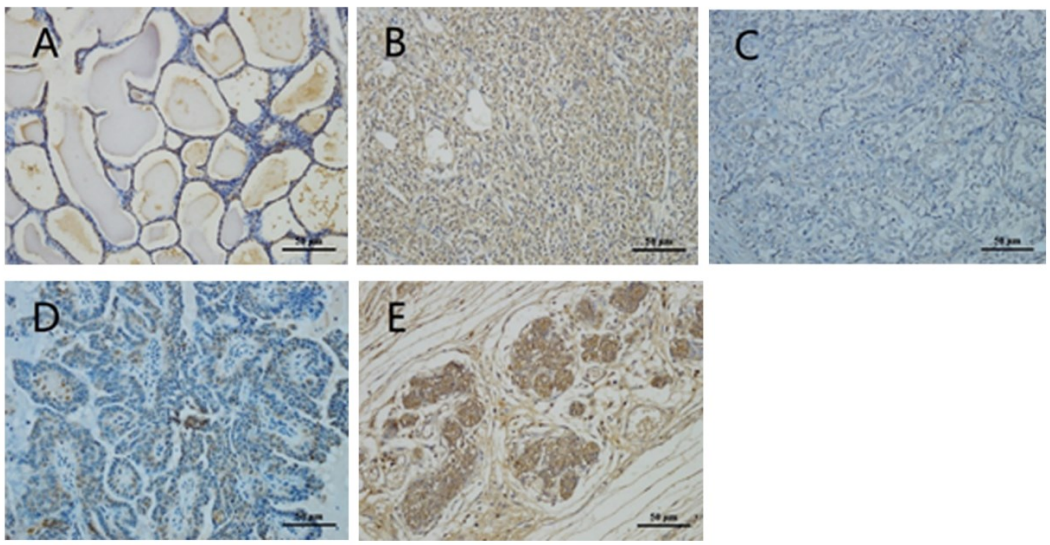

Fig. 2. Expression of the EZH2 protein in normal canine mammary gland and CMC tissue as revealed by immunohistochemical staining (200 ×)
A – normal canine mammary tissues; B – infiltrating ductal carcinoma; C – intraductal papillary carcinomas; D – infiltrating micropapillary carcinoma; E – ductal carcinoma *in situ*. Scale bars: 50 μm

Table 1. Relationship between expression of EZH2 and clinicopathological factors in CMCs

Characteristics	N	Expression of EZH2 protein（n）		P
		-	+	
Age				0.836
＜10	18	8	12	
≥10	35	15	20	
Size (cm)				0.186
＜3	16	9	7	
3–5cm	28	8	20	
≥5	9	4	5	
Histological grade				0.011
I	16	11	5	
II	30	7	23	
III	7	3	4	
Metastatic state				0.424
Yes	12	4	8	
No	41	19	22	
Histological type				0.489
Infiltrating ductal carcinoma	41	12	25	
Intraductal papillary carcinoma	6	4	2	
Invasive micropapillary carcinoma	3	2	1	
Ductal carcinoma *in situ*	3	1	2	

Relationship between EZH2 protein expression and clinicopathological features of CMC. The relationship between EZH2 expression in CMCs and clinicopathological features was further analysed using an χ^2 test. High expression of EZH2 was significantly positively correlated with histological grade (P = 0.011) (Table 1). Compared with grade I, grades II and III had a decreased degree of tumour malignancy, poor prognosis, and increased positive rate of EZH2 expression. However, EZH2 levels were unrelated to age, tumour size, or metastasis. These results suggest that the protein expression of EZH2 in CMC is significantly correlated with the histological grade. In CMC, the higher the histological grade, the higher the EZH2 level and the lower the degree of tumour differentiation.

Discussion

A CMC is a spontaneous tumour occurring in female dogs and is similar to human breast cancer based on pathogenic factors and molecular phenotypes (16, 1). Therefore, studying canine tumours can help identify new cancer-related genes and clarify the molecular pathways; research on them may also bear fruit in new diagnostic, prognostic, and therapeutic tools. Hobert *et al*. (14) discovered the EZH2 gene when studying the proto-oncogene product Vav using the yeast two-hybrid system (13). EZH2 is part of the polycomb repressive complex 2 (PRC2) essential in embryonic development, cell proliferation, and tumour progression (9). This complex catalyses the trimethylation of lysine 27 on histone H3 (H3K27me3) subunit in nucleosomes by assembling around specific gene promoter regions (18). Methylated H3K27me3 silences target genes without modifying DNA sequences and inhibiting gene transcription by recruiting PRC2 to specific gene sites (3). In a study on diffuse intrinsic pontine glioma, it was found that PRC2 activity is required for the proliferation of H3K27M-positive cancer cells and EZH2 inhibitors abolish tumor cell growth and H3K27me3 expression (19), therefore inhibition of EZH2 is a potential therapeutic strategy for the treatment of these tumors.

In the present study, both the EZH2 mRNA and the protein were minimally present in the normal canine mammary tissues. However, 56.6% (30 out of 53) of CMC tissue samples were EZH2 protein positive; the qPCR findings were supported by the immunohistochemical analysis of those sample, suggesting that upregulation of EZH2 may be involved in the progression of some CMCs, which is consistent with other studies. For instance, Choi *et al*. (8) found that EZH2 levels were higher in cancer tissues than in proliferative lesions. Moreover, EZH2 levels are related to the malignancy degree and tumour grade of canine mammary carcinomas (7). Alford *et al.* (2) reported that the positivity rate for EZH2 in breast cancer was 57.6% (277/480). Another study found that 215 out of 432 (49.8%) patients with breast tumours showed high EZH2 expression. In that study, luminal A patients with higher EZH2 levels had shorter overall survival than those with lower EZH2 levels (15). Overexpression of EZH2 occurs in canine lymphoma, melanoma, basal cell tumours, squamous cell carcinoma, histiocytoma, and mast cell tumours, similar to human breast cancer (6). A study has confirmed that its expression is

positively correlated with the invasion of breast cancer cells, and reported that EZH2 knockdown in human inflammatory breast cancer cell lines suppresses the growth of cancer cells, formation of tumour spheres, cell migration and invasion *in vitro*, and angiogenesis and tumour growth in a xenograft model *in vivo* (21). Another study's finding was that EZH2 silencing decreases cell viability and invasiveness, induces apoptosis, and enhances the cytotoxicity of taxanes and cisplatin in Hec-1A and Ishikawa endometrial cancer cells (23). The key finding of our study is that EZH2 deletion, expression, and overexpression occur in normal mammary cells, DCIS, and IDC, respectively, suggesting that EZH2 promotes the evolution of CMC, leading to early carcinogenesis. The homologue is significantly more highly expressed in poorly differentiated DCIS than in precancerous ductal hyperplasia and well-differentiated DCIS. Therefore, it is supposed that EZH2 promotes the development of benign canine mammary gland tumour into poorly differentiated CMCs.

Additionally, we examined the relationship between EZH2 expression and pathological parameters in CMCs. A positive correlation emerged between EZH2 expression and the histological grade and metastasis occurrence of CMCs, similarly to what previous studies found. The gene is significantly increased in presence in triple-negative breast cancer than in benign lesions and normal tissues, which is related to the pathological stage and lymph node metastasis. Additionally, EZH2 presence in breast cancer patients is negatively correlated with relapse-free survival, overall survival, distant metastasis-free survival, and post-progression survival (12). Furthermore, a study involving 96 metastatic breast cancer patients showed that EZH2 expression was higher in metastatic foci than in the primary tumour and was associated with short postoperative OS, a poor prognosis, and tumour recurrence (14). Moreover, EZH2 overexpression was significantly correlated with disease-free and overall survival of patients with endometrial cancer (23). Therefore, dogs with naturally occurring CMCs could be used as an animal model in future clinical trials. These results indicate that EZH2 is often upregulated in cancer cells and may promote the occurrence and development of tumours and that EZH2 is a potential prognostic and therapeutic marker. Disrupting the interaction between EZH2 and other factors may be a potential therapeutic method for CMCs.

In conclusion, abnormal expression of EZH2 may contribute to the tumorigenesis of CMCs. The enhancer of zeste homologue 2 is accumulated mainly in IDC, and it increases in CMCs with high histological grades, especially in histological grade II. Moreover, it correlates with the metastasis state. These findings suggest that the EZH2 level is positively associated with the malignancy degree and could be a potential molecular marker in CMCs diagnosis and therapy.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: Signed informed consents were obtained from all the owners of dogs involved in the present study. All the tissue samples were obtained following veterinary care regulations. The animal experiments were performed following the institutional animal care guidelines and were approved by the Institutional Animal Care and Use Committee of Henan University of Animal Husbandry and Economy, Zhengzhou, China.

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