



## SHORT COMMUNICATION

### Clinico-Pathological, Immunohistochemical and In-Situ TUNEL ASSAY Observations of Canine Breast Tumors

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#### ABSTRACT

Mammary cancer is the most prevalent canine tumor and frequently results in death. This study was carried out for the diagnosis of three canine breast tumors based on clinical, histopathological, immunohistochemistry, and in situ TUNEL assay. Firstly, the morphology of tumor cells was examined by hematoxylin and eosin staining. We found two cases of ductal carcinoma characterized by ductal dilatation and one case of solid carcinoma characterized by extensive connective tissue proliferation. Immunohistochemical assay in paraffin-embedded sections immediately followed surgical removal. The results demonstrated that the expression level of Estrogen receptor (ER), progesterone receptor (PR), p53, vascular endothelial growth factor (VEGF) and proliferating cell nuclear antigen (PCNA) proteins were abnormal. Finally, in situ TUNEL showed a significant decrease in apoptotic cells in canine breast tumors ( $P < 0.01$ ). Taken together, clinical, histopathological, immunohistochemistry, and in situ TUNEL assay will provide a better understanding of the pathogenesis of breast tumors in dogs under field conditions.

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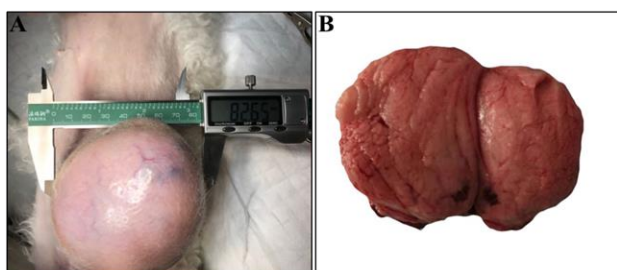
#### INTRODUCTION

Canine breast tumor (CMT) is a naturally occurring heterogeneous, accounting for half of all tumors in female dogs and approximately 40-50% of them are malignant (Kim *et al.*, 2009). Canine breast tumors are the best animal model for studying human breast tumors (Gray *et al.*, 2020). The formation of breast tumors is influenced by multiple factors, and genetic factors are the main cause of tumor formation (Kumar *et al.*, 2007). General clinical examination combined with ultrasound and X-ray examination can almost certainly confirm the diagnosis. However, immunophenotyping has been considered an important tool for cancer classification. Increasing evidence suggests that the use of molecular markers in CMT diagnosis has had a major influence on patient treatment regimens. In dogs, the immunohistochemical

(IHC) assessment of hormone receptors, P53, VEGF, and PCNA has both predictive and prognostic significance, and the changes in expression are associated with clinical outcomes.

#### MATERIALS AND METHODS

Specimens of mammary tumors from four female dogs were collected from a pet hospital in Nanchang, Jiangxi Province. The four dogs comprised two teddy dogs and two poodles with ages ranged from six to 10 years with an average of 8.3 years. Three of the four sick dogs have not been neutered. In addition, the owner told that the sick dogs were long-term weight losers and had a poor mental state. After the dogs were sent to the hospital, we immediately removed the tumor (Fig. 1).



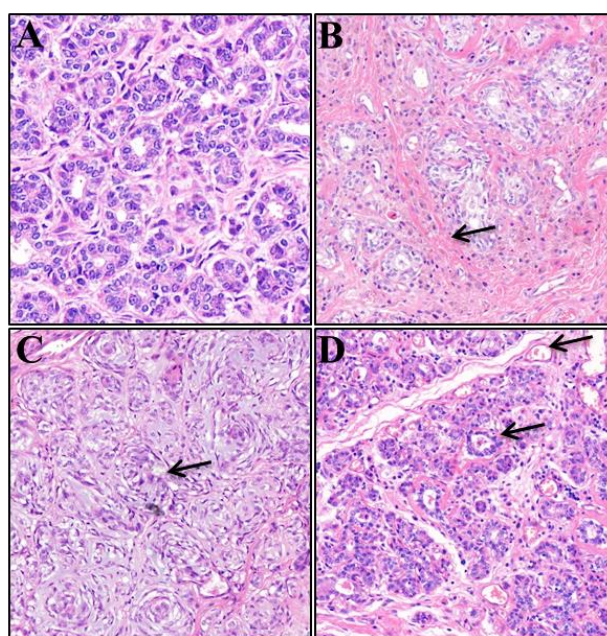
**Fig. 1:** A) There is an 8.3 cm × 7.2 cm mass in the penultimate nipple near the midline of the abdomen in Case 3, and B) Appearance of tumor after surgical removal.

A mass of 8.3×7.2cm penultimate nipple near the midline of the abdomen was present. The fixed breast tissues were fixed in 10% neutral formalin, washed, dehydrated, cleared in xylene, and then waxed. The paraformaldehyde-fixed samples were embedded in paraffin, cut 3-4µm thick sections, and stained with hematoxylin and eosin (Wavumbah *et al.*, 2021). The histopathological slides were then examined under light microscopy (Malak *et al.*, 2020).

For immunohistochemistry, formalin-fixed, paraffin-embedded tissues of 4 µm thick were fixed on slides and deparaffinated and rehydrated with graded ethanol. H<sub>2</sub>O<sub>2</sub> diluted in PBS was utilized for blocking the interference of endogenous peroxidase activity. Microwave reclamation in pH 9.0 Tris-ethylenediamine tetra-acetic acid buffer was applied to the sample for anti-ER, -PR, -P53, -VEGF, and -PCNA. After PBS washing, slides were covered with 3% normal rabbit serum for 30 min to block nonspecific binding. The primary antibody was followed by the secondary antibody-HRP conjugation. Visualization was achieved using DAB1 chromogen (Servicebio Inc., Wuhan, Hubei, China). Finally, distilled water was used for washing of slides, and counterstained with hematoxylin. Six high-power fields were randomly observed under an optical microscope (×400) to observe and record the intensity of cell staining and the proportion of positive cells.

For TUNEL assays, paraffin cut sections were dewaxed, dried and protease K working solution was poured for repair, and afterward, working solution was combined to break the membrane. Reagents 1 (TdT) and 2 (dUTP) are added to the tunnel kit in a ratio of 1:9. The tissues were obscured and divided in a wet box and kept at 37°C for 2 h. The DAPI dye was put in, and the light was evaded and incubated at room temperature for 10 min. Subsequently, DAPI dye was added in a dark environment and incubated at room temperature for 10 min. After washing with PBS solution three times, sections were observed and photographed under a fluorescence microscope. Using fluorescence microscopy, positive cells were examined under three fields randomly selected. The number of effective cells in each visual field and their average fluorescence intensity were calculated, and the mean value was taken as the relative expression of fluorescent immune-positive reactants in the sample.

**Statistical analysis:** SPSS software package (SPSS, Inc., Chicago, IL, USA) was used to analyze quantitatively the collected data for positive staining rates of ER, PR, P53, VEGF, PCNA, and apoptotic positive cells in the breast tumor tissues.

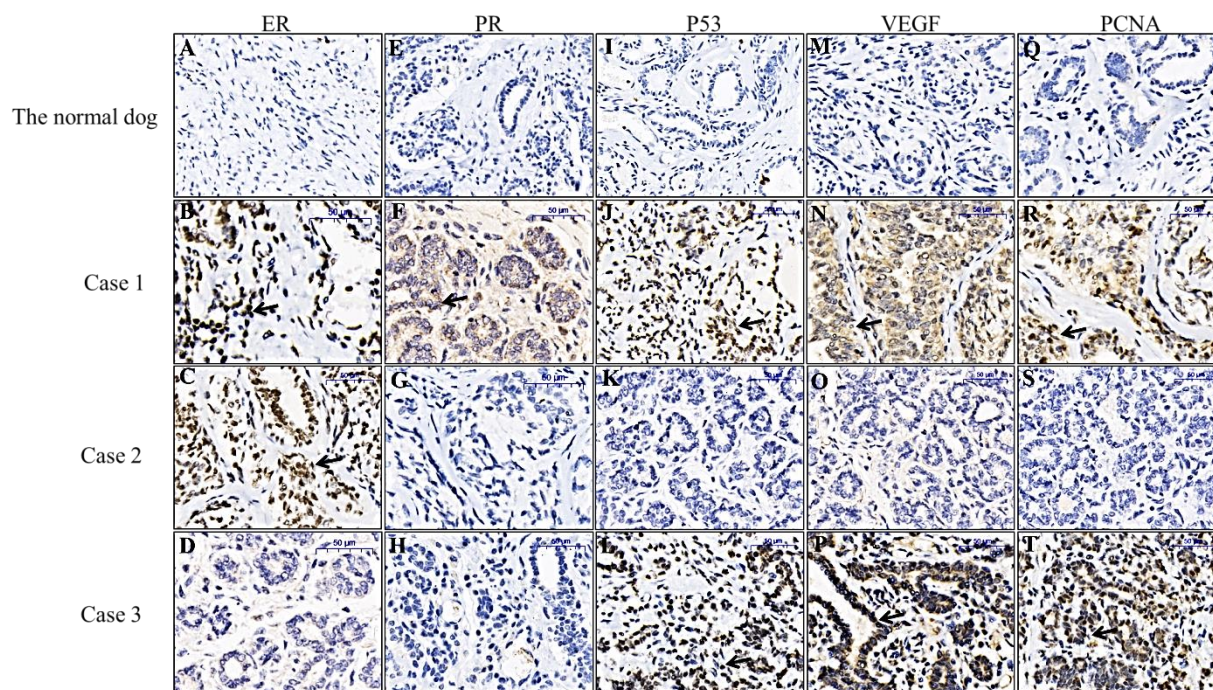


**Fig. 2:** Photomicrograph of canine breast tissue, A) from apparently healthy dog, B and D) Ductal carcinoma and C) Solid carcinoma in canine breast tissue. H&E staining. ×400.

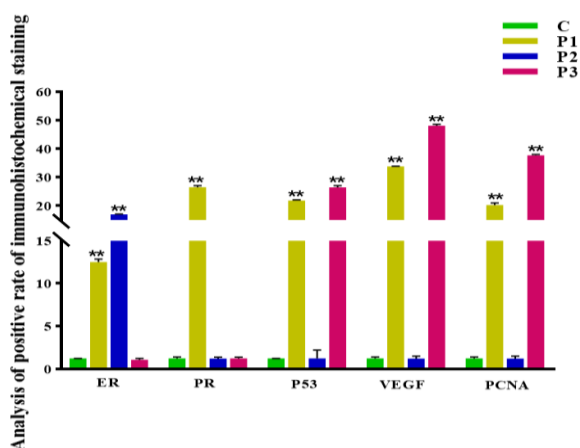
## RESULTS

To determine the nature of breast tumors, the removed breast tumors were embedded in paraffin, followed by H&E staining to observe the tumor tissues. According to the observation of pathological sections (Fig. 2), Case 1 and Case 3 were invasive ductal carcinomas, and Case 2 was solid carcinoma. In Case 1, hyperplastic connective tissue oppresses acini. Acini and mammary ducts vary in size and decrease in number. The breast duct in the tumor is separated by a network of collagen fibers. Case 2 is a solid carcinoma, the mammary duct disappears, and the tumor cells gather to form a solid nest. The tumor cells were clustered and distributed, and the cytoplasm was basophilic in Case 3. The mammary duct is deformed, and the lumen is bleeding.

In addition, the changes of related proteins after the occurrence of breast tumors were analyzed by immunohistochemistry. ER and PR proteins are mainly localized in the nucleus and are clustered or scattered in distribution, especially in solid cancer, where ER and PR are dyed brown and black. P53 protein is mainly located in the nucleus, and P53 protein is scattered in ductal carcinoma. The expression of P53 was not observed in normal breast tissues or solid carcinoma. VEGF and PCNA proteins were not detected in normal breast and solid carcinoma but were strongly positive in ductal carcinoma. VEGF was aggregated and distributed, while PCNA was aggregated or dispersed (Fig. 3-4). To further explore the causes of breast tumors, in-situ TUNEL comparatively analyzed the number of apoptotic cells in diseased dogs (Fig. 5). The percentage of apoptotic cells was calculated in three randomly selected visual fields. The results showed that the number of apoptotic cells decreased significantly in dogs with breast tumors.



**Fig. 3:** The expressions of ER, PR, P53, VEGF and PCNA in breast tissue were detected by immunohistochemistry. Positive expression points are indicated by the black arrow.  $\times 400$ .



**Fig. 4:** Quantitative analysis of positive staining rates of ER, PR, P53, VEGF and PCNA.

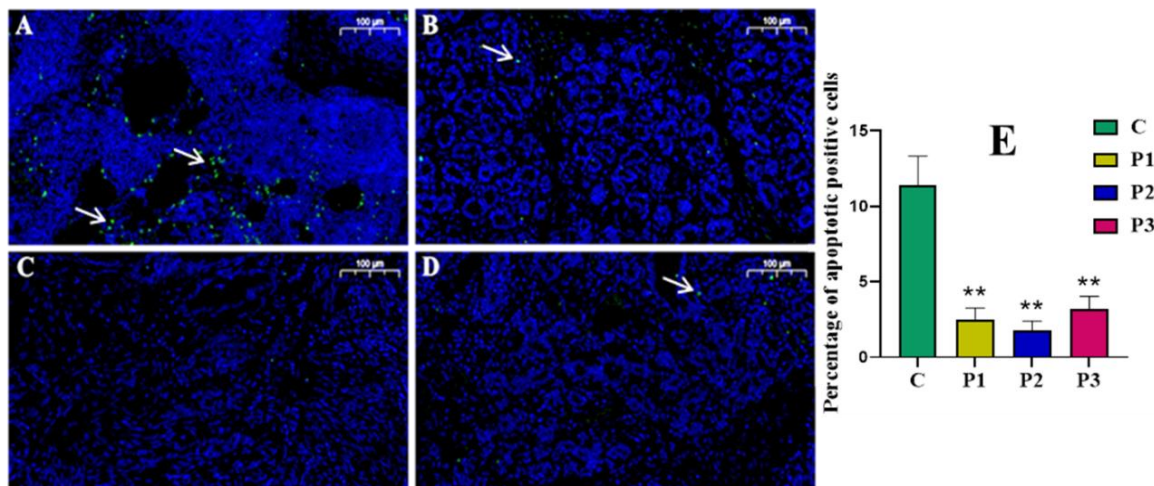
## DISCUSSION

The occurrence of breast neoplasms often is malignant (Eifel *et al.*, 2001), and among the three dogs, there were two cases of ductal carcinoma and one case of solid carcinoma. We immediately performed surgical removal of the affected dogs, one of which died of ductal carcinoma, and the remaining two dogs had a good prognosis.

Immunophenotyping has been considered an important tool for cancer classification. Increasing evidence suggests that the use of molecular markers in CMT diagnosis has had a major influence on patient treatment regimens. It has been widely accepted that estrogen receptor (ER) and progesterone receptor (PR) are used to estimate the possible response to endocrine therapy in patients with breast cancer (Aitken *et al.*, 2010). In the study of three primary breast tumors, it was found that the dogs with ER and PR-negative had a poor

prognosis. Both ER and PR were highly expressed in solid carcinoma. One of the most common tumor suppressor genes in human tumors is the p53 gene. Previous studies have found that mutation of the p53 gene and missense mutation was found in 7 and 5 cases of 20 canine breast tumors, which further confirmed the close relationship between p53 and tumor formation. It has been evidenced that the mutation of the p53 gene results in the inability of p53 protein to control genomic integrity and the inability to induce apoptosis, which leads to the proliferation of damaged cells and then develops into canceration (Flores, 2016).

In our analysis of apoptotic positive cells, we found that the number of apoptotic cells in malignant tumors was significantly reduced, and the relationship between apoptosis and the degree of malignancy of tumors remains to be further studied. The occurrence of abnormal p53 is usually associated with tumor invasion, recurrence, and increased risk of death. In the present study, we found that p53 protein was not detected in benign tumors. P53 protein was heavily expressed in ductal carcinoma and located in the nucleus. The results showed that the expression of p53 protein in breast tumors was specific. The more obvious expression was in the breast tumors with a high malignant degree. According to previous studies, we observed that the expression of VEGF was negatively related to overall survival. In our study, we found that VEGF protein was highly expressed in malignant tumors. Moreover, the expression of VEGF is closely associated with the promotion of angiogenesis and with early relapse in primary breast cancer as well as in other tumors (Ferrara, 2010). This may be since VEGF can promote angiogenesis. As an important part of the DNA replication mechanism, PCNA is an auxiliary protein of DNA polymerase, which can reflect the proliferative activity of tumor cells. Studies have shown that the expression of PCNA varied among breast cancer



**Fig. 5:** A-D indicate apoptosis diagnosed by in-situ TUNEL (green). These pictures are from the normal dog (A), and cases 1-4 (B to D), respectively. E was quantitative analysis of the apoptotic positive cells in breast tumor tissues. Positive expression was pointed out by the black arrow. Magnification: 100 $\times$ .

patients, with mean values ranging from 16 to 46% (Jia *et al.*, 2012). Other reports described a similar direct correlation between the expression of PCNA and p53, which was positively correlated with prognosis. We found that PCNA protein was highly expressed in dogs with poor prognoses. In benign tumors, the expression of PCNA was negative.

Apoptosis is thought to play a key role in the development and growth regulation of normal and neoplastic mammary tissues and its dysregulation is suggested to contribute to the accumulation of mutations that result in mammary tumors (Yang *et al.*, 2006; Pistrutto *et al.*, 2016). Apoptosis is the result of the interaction and co-regulation of apoptotic genes and anti-apoptotic genes. The apoptotic cells in different tissues were detected by in-situ TUNEL. The results showed that the number of apoptotic cells decreased in dogs with breast tumors. In general, inhibition of apoptosis can increase the life cycle of cells and enhance the ability of tumor proliferation and invasion. Case 3 died after surgical excision and was diagnosed as a malignant tumor after histological analysis. By counting apoptotic cells, we found that the number of apoptotic cells was significantly lower than that in the normal dog. Our research and previous studies show that apoptosis is closely related to the occurrence and prognosis of tumors.

**Conclusions:** Histopathological methods are used to make a preliminary diagnosis of breast tumors. The in-depth study of relative proteins and cell apoptosis is of great significance for finding specific molecular markers.

**Authors contribution:** PL planned the study. WC and PL executed the study. YZ, CZ, GL, XG, CH, GH, CW and ZY helped in collection of samples and laboratory work. PL, WC and PL drafted the manuscript while ZX and AK revised it. All authors approved final contents of the manuscript.

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