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Expression of tumour transcription factor GLI1 in canine mammary tumours tissue

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Abstract

Background: Mammary tumor is one of the most common diseases of canine in pet clinics.

Objectives: This study investigates the distribution and expression of the tumor transcription factor GLI1 and the downstream proteins, Bmi1 and Sox2, in canine mammary tumors and paracancerous tissues.

Methods: Cancerous and paracancerous normal mammary tissues were detected using western blotting (WB), and immunohistochemistry.

Results: The results showed that the histopathology of different types in mammary tumors by microscopic observation. GLI1/Bmi1/Sox2 expression was significantly higher in canine mammary invasive carcinoma than in ductal carcinoma and adjacent normal mammary tissues ($p < 0.01$). The expression of GLI1 in invasive carcinoma tissues was significantly higher than Bmi1 and Sox2, while Sox2 expression in ductal carcinoma tissues was significantly higher than GLI1 and Bmi1 ($p < 0.01$). GLI1/Bmi1/Sox2 all showed positive reactions in both mammary tumor and adjacent normal mammary tissues with immunohistochemistry. GLI1 and Sox2 showed strong positive staining in the cytoplasm of invasive mammary carcinoma and ductal carcinoma cells, and weak positive staining in the nuclei. The positive Bmi1 reaction was mainly concentrated in the cytoplasm of invasive carcinoma and ductal carcinoma cells, while the positive reaction on the cell membrane was weak.

Conclusions: We speculate that GLI1 and related proteins play an important role in regulating the proliferation and differentiation of tumors. Therefore, it provides important reference for the pathogenesis and pathogenicity of canine mammary tumor.

KEYWORDS

canine, expression, GLI1/Bmi1/Sox2, mammary tumours

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1 | INTRODUCTION

Canine mammary tumours are the most common tumours in female dogs, accounting for more than 50% of the cases. The incidence rate of canine mammary tumours represents 5%–10% of all canine diseases. Purebred canines are more susceptible to develop them, such as teddy, sheriffs, pit bulls, Alaskans, and so on (China Industrial Research Institute, 2021), and the incidence rate increases gradually with age. After clinical diagnosis, histological diagnosis after surgical removal of malignant tumour combined with drug therapy is the best treatment. Clinical observation showed that ductal carcinoma and invasive carcinoma were the most common types of mammary tumours in canine (Canadas et al., 2019). With this treatment regime, the secondary recurrence rate of malignant tumours exceeds 40% within 2 years (Alonso et al., 2021). In addition, this cancer has reference significance for the research and prevention and treatment of human breast tumours (Raposo et al., 2017). Therefore, exploring the pathogenesis of canine mammary tumours and reducing the recurrence rate are the focus of considerable research.

Canine mammary tumour occurrence is regulated by p53, Wnt, and other related tumour signalling pathways; increased expression of p27 and p53 prevents the proliferation of canine breast tumours (Tong et al., 2016). Genomics and precision medicine research have shown that the mutations and aberrations induce the development of human and canine mammary tumours, and the key regulatory genes are very similar (Kim et al., 2020). Therefore, the study of the specific expression of canine breast tumour regulatory factors and stem cell markers provides an important scientific basis for revealing the occurrence and metastasis of canine breast tumours.

GLI1 is the most important transcription factor of the Hedgehog (Hh) signalling pathway. The sonic hedgehog-GLI1 (shh-GLI1) pathway is abnormally activated in human breast, liver, pancreatic, and colon cancer, and can stimulate the proliferation, differentiation, apoptosis, and angiogenesis of cancer cells (Jeng et al., 2020). High expression of GLI1 promotes the carcinogenesis of basal epithelial cells in breast tissue and enhances the proliferation, invasiveness, and self-renewal capability of breast cancer stem cells. The downstream core proteins of GLI1, Bmi1, and Sox2 are directly involved in the self-renewal of breast cancer stem cells (Marzagalli et al., 2021). The ubiquitous nature of Bmi1 in breast cancer tissues and cells is associated with their proliferation and metastasis (Witus et al., 2021). In addition, the activation of Sox2 by STAT3 promotes the anti-apoptotic and growth capabilities of stem cells (Dittmer & Dittmer et al., 2020), while the proliferation and migration ability of Sox2 knockdown canine breast tumour CHMm cells decreased significantly (S. Wang et al., 2020). Therefore, it was confirmed that GLI1 is an important protein of the shh-GLI1 pathway during the invasion and metastasis of breast tumour cells.

In this study, protein expression of GLI1/Bmi1/Sox2 in canine breast tumour tissues and adjacent tissues was investigated using western blotting, immunohistochemistry, and immunofluorescence to provide an experimental basis for exploring the shh-GLI1 signalling pathway regulating the proliferation and differentiation of canine breast tumour cells.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and materials

This experiment was approved by the institutional review board of Animal Ethics Committee of Gansu Yangzhou University (SYXK(Su)2017-0044). Samples were collected from poodle and bulldog in the animal hospital of Yangzhou University from September 2018 to August 2020. We collected six dogs with mammary tumours, and paraffin-embedded tumour blocks were obtained from three dogs with invasive tumours and three ductal carcinoma and used for immunohistochemical staining (Goldschcmidt et al., 2011). The surgically removed canine breast tumour tissue samples were trimmed to 1 cm^3 and fixed in 4% paraformaldehyde solution. The pathological tissue sections were prepared and further confirmed by haematoxylin and eosin (HE) staining and necrosis in the lumen; the primary ductal carcinoma had interstitial hyperplasia and lobulated. Sections were prepared for immunohistochemistry and immunofluorescence, and the remaining tissues were stored at −80°C.

2.2 | Western Blot

Breast invasive carcinoma, primary ductal carcinoma, and adjacent tissues were weighed, and lysed in 1 ml RIPA buffer and 10 ml RIPA buffer, respectively. Then 10 μl PMSF was added and placed on a shaking table for 2 h at 4°C, and then centrifuged at 12,000 × g for 12 min, and the supernatant was removed for later use. The extracted protein sample was denatured with 6× loading buffer at 95°C for 8 min.

According to the principle of random sampling, the prepared experimental tissues were selected from three dogs with invasive tumours: ductal carcinoma and adjacent normal mammary tissues. The samples were transferred from an SDS-PAGE gel to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). After rapid blocking, the membrane was probed with rabbit polyclonal antibody GLI1 (bs-1206R, Bioss, 1:400 dilution), rabbit polyclonal antibody Bmi1 (bs-2999R, Bioss, 1:500 dilution), and mouse monoclonal antibody Sox2 (bs-0523R, Bioss, 1:500 dilution) at 4°C overnight. After primary antibody incubation, the membrane was washed with TBST three times for 3 min each, and probed with HRP-labelled secondary antibody (1:1000 dilution) at room temperature (24°C) for 1.5 h. The membrane was washed with TBST (three times, 3 min each), and then ECL Kit (NCM Biotechnology, China) was used to detect the protein bands for GLI1/Bmi1/Sox2. Finally, the optical density of the imprinted bands was measured by optical density analysis (Bio-Rad) using GAPDH as a reference (Sirkisoon et al., 2018).

2.3 | Detection of GLI1/Bmi1/Sox2 by immunohistochemistry

Invasive mammary tumours (three cases), ductal carcinoma (three cases), and paracancerous normal mammary (three cases) tissues in

dogs were fixed with 4% paraformaldehyde solution at room temperature. Tissue blocks for the experiment were selected, embedded in paraffin, sliced, dried, and stored at 4°C. HE staining section of breast tumour tissue was performed and observed. To detect the positive immunohistochemical reaction of GLI1/Bmi1/Sox2, all the previous experimental steps were completed in strict accordance with the immunohistochemical operation procedures. The samples were then soaked with 3% deionized H_2O_2 for 15–20 min, sealed with goat serum for 15–20 min, treated with monoclonal antibodies (GLI1, Sheep anti mouse; Bmi1, Sheep anti rabbit; and Sox2, Sheep anti mouse) (1:300, Abcam, Hong Kong), and incubated overnight at 4°C. After washing, corresponding secondary antibody was added dropwise for incubation (Goat anti mouse IgG (H + L)/HRP, bs-40296, Bioss, 1:3000 dilution; Goat anti rabbit IgG (H + L)/HRP, bs-40295G, Bioss, 1:3000 dilution). The reaction-labelled samples were preferably counterstained with 3-3'-diaminobenzidine (Sirkisoon et al., 2018). In addition, immunohistochemistry and immunofluorescence tests were negative control.

2.4 | Positive detection of GLI1/Bmi1/Sox2 immunofluorescence

For immunofluorescence, secondary antibodies were not added for incubation, but fluorescent secondary antibodies (goat anti-mouse IgG (H + L), bs-40296G-IRDye8, Bioss, 1:800 dilution; goat anti-rabbit IgG (H + L), bs-40295G-IRDye8, Bioss, 1:800 dilution) were incubated for 2 h and observed by sealing.

2.5 | Statistical analysis

The results of western blotting, immunohistochemistry, and immunofluorescence were measured using Image-Pro Plus 6.0. The experimental data were evaluated using spss21.0 software. The final results were expressed as the mean ± standard deviation. The differences between samples were analyzed by an independent sample *t*-test (one-way analysis of variance, least significant difference [LSD]). The differences between the different groups of samples were analyzed using the LSD method. $p < 0.05$ indicates significant difference; $p > 0.05$ indicates that the difference is not significant (Oladapo et al., 2017).

3 | RESULTS

3.1 | Histopathological observation of canine mammary tumours

Histopathology of different types in mammary tumours by microscopic observation (a) 10×; (a-1) 20×; (a-2) 40×. As shown in Figure 1, histopathological observation revealed extensive epithelial hyperplasia in the infiltrating carcinoma, some of which penetrate the basement membrane into the connective tissue. Ductal epithelial hyperplasia of the carcinoma, forming multiple papillary structures with fibrous vascular bundles, was judged as ductal carcinoma.

3.2 | Expression of GLI1/Bmi1/Sox2 in canine mammary tumours

There was no difference in the expression of GLI1/Bmi1/Sox2 proteins within the same carcinoma types of different canine breeds, but there was a significant difference between the expression of GLI1/Bmi1/Sox2 protein in different types of carcinoma and adjacent normal tissues ($p < 0.01$). As shown in Figure 2, the expression of GLI1/Bmi1/Sox2 in canine invasive mammary tumours was significantly higher than that in ductal carcinoma and adjacent normal mammary tissues, and the expression in ductal carcinoma was significantly higher than that in adjacent normal mammary tissues ($p < 0.01$). The expression of GLI1 in invasive mammary tissues was significantly higher than that in Bmi1 and Sox2, whereas the expression of Sox2 in ductal carcinoma tissues was significantly higher than that of GLI1 and Bmi1. The expression of Bmi1 was the lowest in the two tumours types, and there was a significant difference between them ($p < 0.01$), as shown in Figure 2.

3.3 | Positive expression of GLI1/Bmi1/Sox2 immunohistochemistry

Immunohistochemistry in canine mammary carcinomas and adjacent normal mammary tissues were positive for GLI1/Bmi1/Sox2. GLI1/Sox2 had a strongly positive reaction in the cytoplasm and weakly positive in the nuclei of mammary invasive carcinoma and ductal carcinoma cells. The positive reaction of Bmi1 was mainly concentrated in the cytoplasm of invasive carcinoma and ductal carcinoma cells, while the positive reaction was weak on the cell membrane (as shown in Figure 3a,b). Immunohistochemical optical density analysis showed that the positive expression of GLI1/Bmi1/Sox2 was the highest in invasive carcinoma, followed by ductal carcinoma, and the lowest in adjacent normal mammary tissues. The expression of GLI1 was the strongest in invasive carcinoma ($p < 0.01$), and there was no significant difference for Bmi1/Sox2. The positive expression of Sox2 was the strongest in ductal carcinoma ($p < 0.01$), and there was no significant difference in GLI1/Bmi1. There was no significant expression in the adjacent normal mammary tissues (Figure 3c).

3.4 | GLI1/Bmi1/Sox2 immunofluorescence reaction

Immunofluorescence observation showed a positive reaction for GLI1/Bmi1/Sox2 in canine mammary carcinomas and adjacent normal mammary tissues (Figure 4a–c). The GLI1/Sox2 positive reaction was stronger in the cytoplasm of invasive carcinoma and ductal carcinoma cells, with a small amount of positive staining in the nucleus; Bmi1 was mainly expressed in the cytoplasm of invasive carcinoma and ductal

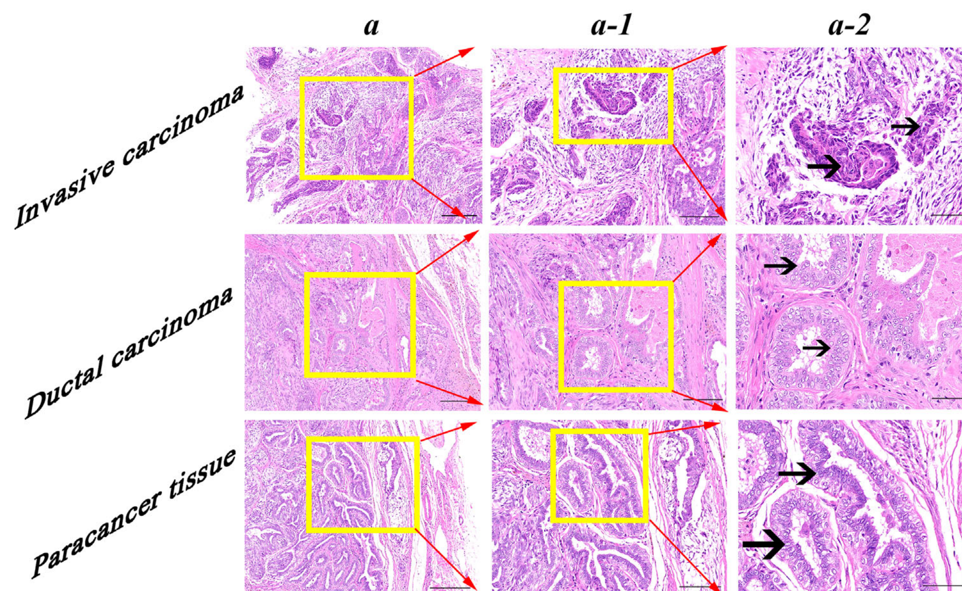

FIGURE 1 Histopathological observation of canine breast tumours. Histopathological haematoxylin and eosin (HE) section of mammary tumour was used to observe the results. (a) 10×; (a-1) 20×; (a-2) 40×

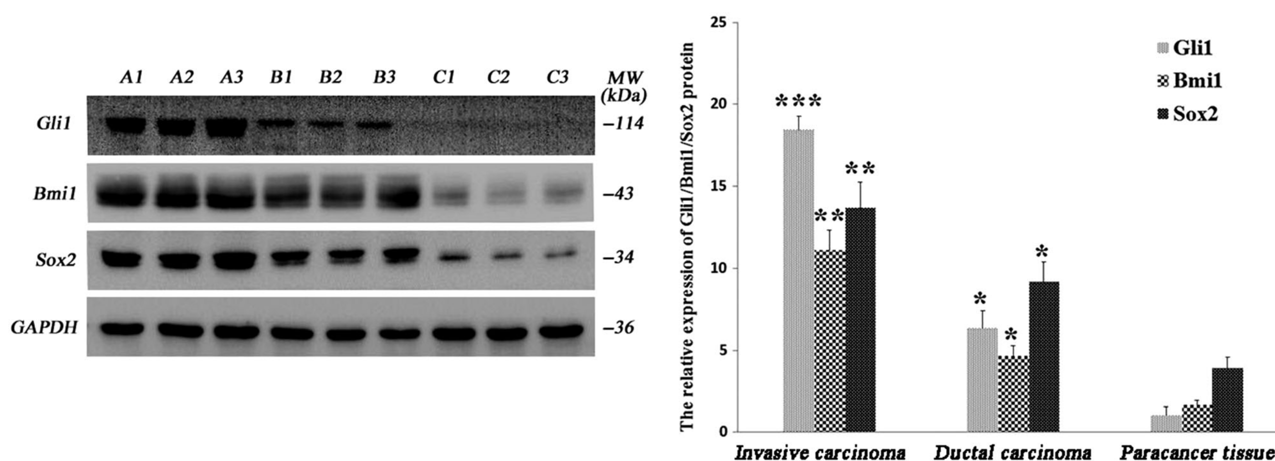

FIGURE 2 Expression of GLI1/Bmi1/Sox2 protein in canine mammary tumours and adjacent normal mammary tissues. Western blot results for GLI1/Bmi1/Sox2 protein. Grey value analysis of GLI1/Bmi1/Sox2 protein expression. ***Very significantly different ($p < 0.01$), **significantly different ($p < 0.05$), *not significantly different ($p > 0.05$). A Invasive carcinoma, B ductal carcinoma, C adjacent normal mammary tissues; 123 samples from three different canines

carcinoma cells, and there was a weak positive reaction on the cell membrane. At the same time, IgG was selected as the negative control, and no positive reaction was observed, as shown in Figure 4. In addition, semi-quantitative analysis of immunofluorescence results showed that the positive expression of GLI1/Bmi1/Sox2 was consistent with the immunohistochemical results, as shown in Figure 4d.

4 | DISCUSSION

Here, we report for the first time the expression and localization of GLI, Sox2, and Bmi1 in canine mammary tumours. GLI1 is the most important transcription factor of the Hh signalling pathway. The abnormal activation of the shh-GLI1 signalling pathway can stimulate the proliferation, differentiation, apoptosis, invasion, and metastasis of tumour cells and the formation of new blood vessels (Doheny et al., 2020). Previous studies have shown that the expression of Nanog, CD44, CA15-3, and SRC-3 in canine mammary tumours tissues is significantly higher than that in healthy mammary glands. These studies suggest that these proteins are likely to be key regulatory factors and markers of canine mammary tumour stem cells (Costa et al., 2019; H. Wang et al., 2019). The expression of GLI1 in human invasive breast ductal carcinoma was significantly higher than that in adjacent tissues, and the activation of GLI1 not only promoted the proliferation of breast tumour cells

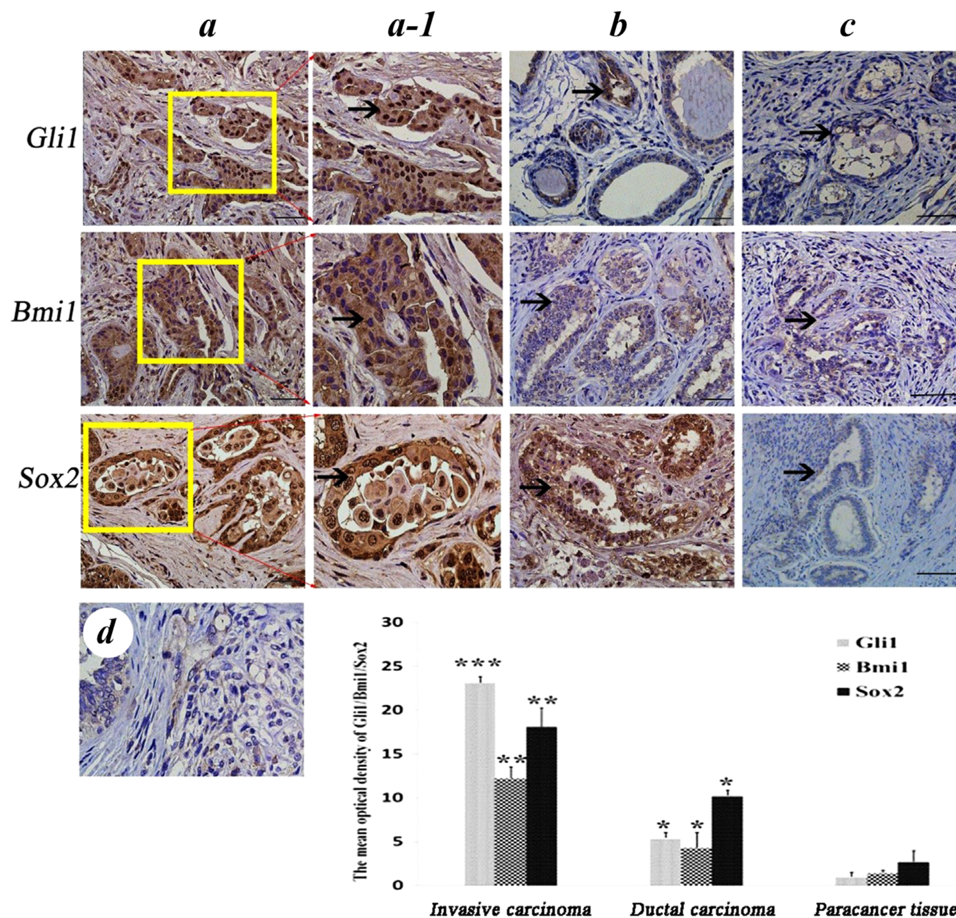

FIGURE 3 GLI1/Bmi1/Sox2 were positively co-expressed in canine mammary tumours and adjacent normal mammary tissues. (a) Invasive carcinoma; (a-1) enlarged view of invasive carcinoma; (b) ductal carcinoma; (c) adjacent normal mammary tissues; (d) negative control; grey value analysis results. →, GLI1/Bmi1/Sox2 positive reaction site

but also significantly enhanced invasiveness (Jeng et al., 2014). The results showed that the expression of GLI1 protein in canine invasive breast cancer and ductal primary carcinoma was significantly higher than that in adjacent tissues, indicating that GLI1 is directly or indirectly involved in the proliferation and invasion of canine breast cancer cells.

In human breast tumour tissues, the positive expression of tumour tissues was significantly higher than that of adjacent tissues, but there was no significant correlation with patient age, tumour size, and pathological grade (Xu et al., 2016). Bmi1 and Sox2, as downstream core proteins of the GLI1 signalling pathway, are directly involved in the regulation of tumorigenesis and development in human. The downregulation of Bmi1 can significantly inhibit the formation of prostate tumours in mice and the growth, proliferation, and invasion of prostate tumour cells in vitro, while the upregulation of Bmi1 can promote the formation of cancer cell microspheres and cell migration (Jin, 2014; Lu et al., 2021). In addition, the overexpression of Sox2 promotes the invasiveness of glioma cells, while low expression and knockdown of Sox2 promotes the proliferation of tumour cells, and formation of microspheres, metastasis, and invasion of tumour cells significantly reduced (Schaefer et al., 2019; S. Wang et al., 2020). It was found that the expression of Bmi1 and Sox2 proteins in canine invasive breast cancer and ductal primary cancer was significantly higher than those in adjacent tissues. Based on the above results, we hypothesized that Bmi1 and Sox2 may directly or indirectly participate in the proliferation and invasion of canine breast cancer cells.

GLI1 protein was mainly stained in the nuclei and cytoplasm in breast cancer tissue, ranging from pale yellow to dark brown. It was mostly stained in the cytoplasm in the paracancerous tissue, followed by the nucleus (Zhang, 2019). In this study, GLI1 and Sox2 had a strongly positive reaction in the cytoplasm and weakly positive in the nuclei of mammary invasive carcinoma cancer and ductal carcinoma cells; Bmi1 was mainly distributed in the cytoplasm with a small amount of positive distribution in the cell membrane. Human breast tumour research found that the positive rate of GLI1 expression in breast cancer adjacent tissues was 20%. The positive rate of expression in benign breast lesions was 56.4%, and the positive rate of expression in breast invasive ductal carcinoma was 38.7% (Quan, 2015). In addition, the high expression of Sox2 and Bmi1 in tumour cells promotes the occurrence and development of breast cancer. Inhibition of Sox2 reduced cell proliferation and migration and increased apoptosis. The positive reaction was concentrated in the cytoplasm of tumour cells

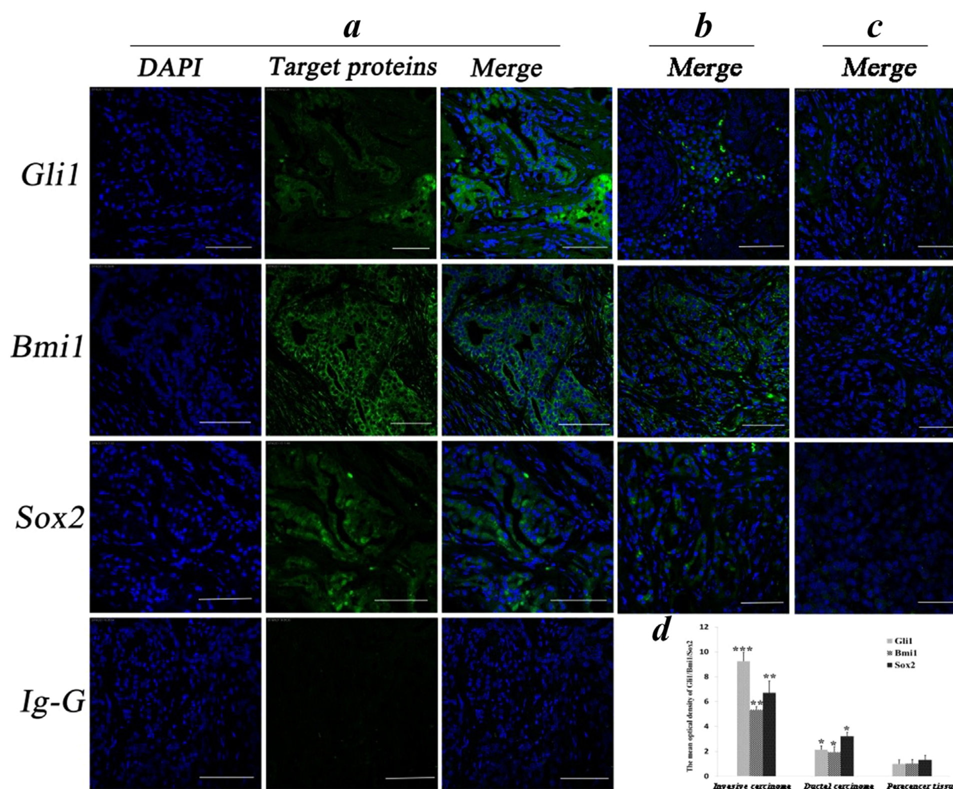

FIGURE 4 GLI1/Bmi1/Sox2 were positively co-expressed in canine mammary tumours and adjacent normal mammary tissues. (a) Invasive carcinoma; (b) ductal carcinoma; (c) adjacent normal mammary tissues; Ig-G is positive control. Optical density analysis results. DAPI is nuclear staining, target proteins are target protein staining, and merge is the synthesis diagram

(Hütz et al., 2014; Yang et al., 2019). To summarize the relevant research, we conclude that GLI1 protein not only activates the carcinogenesis of breast cells but also promotes the typing of tumours. Furthermore, Bmi1 and Sox2 play synergistic roles in tumour formation.

5 | CONCLUSION

The expression of GLI1/Bmi1/Sox2 in canine mammary tumours and adjacent normal mammary tissues was significantly different. The positive reaction was mainly concentrated in the cytoplasm of tumour cells. We speculate that the tumour transcription factor GLI1 and related proteins play an important role in regulating the proliferation and differentiation of canine mammary tumours.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Penggang Liu, Yurong Lu, and Jing Sun designed the experiments, performed the experiments, analyzed and interpreted data, wrote, and edited the manuscript. Penggang Liu, Yurong Lu, and Xueli Chen performed the experiments, analyzed, and interpreted data in the manuscript. Yang Yang and Wenbing Xiong contributed reagents/materials/analysis tools for manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [author initials], upon reasonable request.

PEER REVIEW

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