Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.262

RESEARCH ARTICLE

Establishment and Characterization of a New Canine Mammary Cancer Cell Line CMT-N7: Implications for Comparative Oncology and Therapeutic Development

Xinhao Song[1,2], Shasha Gao[1,2], Shiheng Hu[1,2], Tian Fang[1,2], Xiaolin Xu[1,2], Xin Lv[1,2], Xiuge Gao[1,2], Mengjuan Lin[1,2], Lin Peng[1,2], Meng Li[1,2], Yingjun Lv[1,2], Shanxiang Jiang[1,2] and Dawei Guo[1,2*]

[1]Engineering Center of Innovative Veterinary Drugs, Center for Veterinary Drug Research and Evaluation, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, China
[2] MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, China
*Corresponding author: gdawei0123@njau.edu.cn

ARTICLE HISTORY (24-272)

Received: May 15, 2024
Revised: September 19, 2024
Accepted: September 20, 2024
Published online: October 11, 2024

Key words:
Canine mammary tumor
Cell line
Epithelial-mesenchymal transition

ABSTRACT

Canine mammary tumor (CMT) is one of the relevant models of human breast cancer (HBC) with histopathological, epidemiological, and clinical characteristics similar to those of humans. This study aimed to establish and characterize a new canine cell line CMT-N7. CMT-N7 tumor is a complex canine mammary carcinoma that stained negative for human epidermal growth receptor-2 (HER2) and progesterone receptors (PR), and positive to estrogen receptor (ER). Cell growth, ultrastructure, doubling time, metastasis capacity, and biomarker characteristics of CMT-N7 were assessed. Xenograft transplantation was conducted to evaluate tumorigenicity. The cell morphology of CMT-N7 was generally epithelioid, with large and irregular nuclei and obvious multinucleation. The established CMT-N7 cell line underwent over 120 generations of subculture, exhibiting a rapid proliferation rate with a doubling time of 20.34 h and a chromosome number ranging from 70 to 90. Transwell and wound healing assays demonstrated the CMT-N7 cells had invasive ability. Immunofluorescence analysis revealed positive expression of ER, α-SMA, CK-14, SOX-2, Vimentin, Ki-67, E-cadherin, and COX-2 in CMT-N7 cells. Following inoculation with CMT-N7 cells for two weeks, all mice developed tumors. Immunohistochemical analysis showed negative expression of HER-2 and PR, and positive expression of ER, Ki-67, E-cadherin, Vimentin, and COX-2. Consequently, the establishment of the canine mammary cancer cell line CMT-N7 provides a good model for investigating the mechanism of epithelial-mesenchymal transition (EMT) in both dogs and humans.

To Cite This Article: Song X, Gao S, Hu S, Fang T, Xu X, Lv X, Gao X, Lin M, Peng L, Li M, Lv Y, Jiang S and Guo D, 2024. Establishment and characterization of a new canine mammary cancer cell line cmt-n7: implications for comparative oncology and therapeutic development. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2024.262

INTRODUCTION

Canine mammary tumors (CMT) are highly prevalent in aged, unsterilized female dogs with a significant morbidity and mortality rate, and account for approximately 50% of all the female canine tumors encountered in veterinary clinics (Srisawat *et al*., 2020; Ou *et al*., 2021). Extensive research has consistently demonstrated the remarkable similarities between CMT and human breast cancer (HBC) in terms of clinicopathological characteristics, epidemiology, and pathogenesis (Rivera and von Euler, 2011; Markkanen, 2019). The shared characteristics between these two entities facilitate comparative oncology research. In addition, they hold promising implications for advancing our understanding of the underlying mechanisms and potential therapeutic strategies for both canine and human breast cancer. An authoritative report reveals a remarkable degree of similarity between canine and human gene sequences that surpasses those found in rodent genomes (Lindblad-Toh *et al*., 2005). CMT, as a model of spontaneity, is the natural occurrence of mammary tumors in dogs without any external intervention or manipulation by researchers. This means that tumors arise in dogs due to their genes, the environment, or a combination of both, just like in humans. Thus, CMT can serve as a valuable spontaneous animal model for investigating HBC. The accelerated incidence and progression of mammary

tumors in dogs, due to their shorter lifespan and increased number of estrous cycles, provide a valuable platform for investigating various aspects of HBC, including mechanisms of tumorigenesis, heredity and epigenetics, and biological characteristics of tumors (De la Roca-Chiapas *et al*., 2016).

Tumor cell culture plays a pivotal role in cancer research for investigating the intricate mechanisms underlying cancer development. It provides a robust platform for conducting comprehensive exploration into the functional properties of tumor cells, and their influential factors, elucidating carcinogenesis mechanism, comprehending molecular biological characteristics, and efficiently screening potential anticancer drugs. By establishing and maintaining tumor cell lines, we can overcome the limitations associated with individual patient variability and the dynamic internal environment of the body. Therefore, the in vitro culture of tumor cells holds significant importance in advancing basic research. Immortalized tumor cell lines are considered an ideal experimental model (Esparza-López *et al*., 2019). However, it is a challenging task to establish immortalized tumor cell lines successfully during the process of in vitro isolation and culture of tumor cells. In the initial stages of culture, tumor cells exhibit a slow rate of growth and proliferation. In addition, they are susceptible to the influence exerted by other cell types, such as fibroblasts, resulting in growth disadvantages for the tumor cells. Moreover, the biological and genetic characteristics of tumor cells may undergo alterations over time due to prolonged culture, repeated passages, or changes in the culture environment. Hence, persistently isolating and culturing primary tumor cells from solid tumors to create new cell lines is vital for ensuring experimental cancer models' relevance and accuracy.

Despite the recent establishment of several canine mammary gland cancer cell lines, their limited numbers and varying characteristics necessitate the urgent establishment of new lines that adequately represent the diverse features of original mammary tumors. In light of this, our research effectively developed a new CMT cell line and thoroughly analyzed its morphology, growth characteristics, karyotype, and protein profile. This investigation introduced an exceptional in vitro model that holds promise for advancing research in breast cancer treatment.

MATERIALS AND METHODS

Establishment of CMT-N7 cell line: Mammary tumor tissues from dogs with mammary gland tumors were aseptically excised in Nanjing Agricultural University's Veterinary Hospital. These tissues were promptly immersed in serum-free DMEM medium (HyClone, USA) containing antibiotics. After PBS washing and fragmentation, the tissues were digested in 0.1% collagenase II (Gibco, USA) at 37°C for 1 h. The resulting cell suspension was filtered, centrifuged, and resuspended in a complete DMEM medium with 10% FBS (Gibco, USA) and antibiotics. Cells were cultured at 37℃ and CO_2, and passaged in fresh DMEM-10% FBS medium at a 1:3 ratio every 2-3 days.

Transmission electron microscopy assay: Transmission electron microscopy assay was conducted as described previously (Lin *et al*., 2023). Upon reaching 80%-90% confluency, the CMT-N7 cells were harvested, centrifuged at 1000 rpm for 4 min, and then fixed by adding 2.5% glutaraldehyde solution and placed at 4℃ for overnight fixation. Subsequently, washing with PBS and then dehydration was carried out using graded ethanol solution (30%, 50%, 70%, 80% and 100%). To stain the cells, lead citrate and uranyl acetate were utilized and photographs were observed.

Growth assay: Evaluation of the growth curve at higher levels provides insight into cells' growth characteristics and stability after prolonged culture. Thus, the growth curve was established upon stable cultivation of CMT-N7 cells up to 52 passages. Cells were diluted to a series of concentrations that allowed to easily prepare the standard curve as previously described (Zhang *et al*., 2018). After being diluted to varying concentrations in a complete DMEM medium, cells in an ideal growth environment were evenly distributed into 96-well plates with six wells for each concentration. After 24 h incubation, CMT-N7 cells were treated with CCK-8 (Beyotime, Shanghai, China) in triplicate for 1 h and subsequently monitored at 24 h intervals for a total of 10 d.

Karyotype analysis: To perform karyotype analysis, CMT-N7 cells in the stage of rapid growth were exposed to 0.06 mg/mL colchicine for a duration of 6 h. Subsequently, the cells were immersed in a 0.075 M KCl solution at a temperature of 37℃ for 40 min before being preserved using a combination of methanol and glacial acetic acid (3:1, v/v). The cell suspension was carefully placed onto a chilled slide, followed by staining with Giemsa stain (Solarbio, Beijing, China) for approximately 10 min. Following the removal of the staining solution, the chromosomes were viewed and tallied.

Mycoplasma detection: Mycoplasma DNA was detected with a GMyc-PCR Mycoplasma Test Kit (Yeasen, Shanghai, China) according to the manufacturer's instructions. Subsequently, the PCR samples were analyzed by electrophoresis on a 0.8% agarose gel.

Wound healing assay: The wound-healing assay was conducted as described previously (Song *et al*., 2023). Briefly, CMT-N7 cells were seeded at a density of 1×10^5 cells/well in 6-well plates and their motility was evaluated by measuring their movement towards a steep slope. The speed of scarp closure was monitored after 24 h by calculating the ratio of the distance of the scarp from 0 h. The microscopic scratch wound area of each plate was photographed by performing an inverted phase-contrast microscope (Leica, Wetzlar, Germany).

Invasion assay: For the transwell assay, CMT-N7 cells (2×10^4 cells per well) were placed in matrigel (Corning Incorporated, New York, USA) coated bottom chambers. Medium plus 10% FBS was added in the lower chamber as the chemo-attractant. After 24 h, the inserts were removed, and the cells on the surface of the bottom chambers were fixed with 4% paraformaldehyde,

followed by staining with 0.5% crystal violet. The cells in microscopic fields were counted and photographed using an inverted phase-contrast microscope (Leica, Wetzlar, Germany).

Indirect immunofluorescence analysis (IFA): CMT-N7 cells were seeded in 24-well plates (1×10^5 cells/well) and incubated overnight. Cells were fixed with 4% paraformaldehyde for 30 min, washed thrice with PBS, and blocked with goat serum for 30 min. Cells were incubated overnight at 4°C with primary antibodies targeting ER (Servicebio, GB14080, 1:500), E-cadherin (Servicebio, GB12082, 1:500), CK-14 (Servicebio, GB11803, 1:500), Vimentin (Servicebio, GB111308, 1:500), Ki-67 (Servicebio, GB111141, 1:500), COX-2 (Servicebio, GB115672, 1:500), α-SMA (Servicebio, GB111364, 1:500), and SOX-2 (Servicebio, GB11249, 1:500). After PBST washing, cells were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Servicebio, GB28301, 1:200) for 1 h. Cells were then stained with DAPI for 10 min and observed under a fluorescence microscope.

Tumorigenicity in nude mice: Animal experiments were carried out according to the Guidelines for Animal Experimentation of Nanjing Agricultural University, and the protocol was approved by the Animal Ethics Committee of this institution (No.20220930184). Female BALB/c nude mice (4~5 weeks old) were obtained from Yangzhou University (Yangzhou, China), and fed in the Laboratory Animal Center of Nanjing Agricultural University (Nanjing, China).

The cells were subcutaneously inoculated into the left mammary fat pad of mice at a density of 1×10^6 cells per mouse (n=10). Tumor growth was monitored weekly in mice. Once tumors were identified, they underwent weekly monitoring through palpation and caliper measurements. After sixty days following inoculation, mice were humanely euthanized via cervical dislocation. Tumors were then collected and fixed in a 4% paraformaldehyde solution to create wax blocks for subsequent histological and immunohistochemical examinations.

Hematoxylin-eosin and immunohistochemistry staining: For histopathological assessment, representative tumors with lesions were fixed in 10% buffered formalin, sectioned into 4-μm-thick slices, subsequently dehydrated in a series of graded ethanol, and stained with hematoxylin and eosin (H&E).

For immunohistochemical analysis, paraffin-embedded specimens were sectioned to 4 μm, dehydrated, and antigen-retrieved at 98°C. After cooling, slides were treated with 3% H_2O_2, blocked with 5% goat serum, and incubated with primary antibodies (ER, PR, HER-2, E-cadherin, Vimentin, Ki-67, COX-2) overnight at 4°C, and the sections were then placed in PBS and washed by shaking on the decolorization shaker. After the sections were gently shaken and dried, the tissues were coated with Alexa Fluor 594 goat anti-rabbit IgG for incubation for 30 min. After PBS washes, sections were stained with DAB, counterstained with hematoxylin, dehydrated, cleared with xylene, and mounted. Each section was examined and photographed under a light microscope (CX23, OLYMPUS) for histopathology analysis.

Statistical analyses: Results were shown as mean ± standard deviation (SD) in this study. Statistical analyses were carried out using one-way ANOVA followed by a Tukey's test. GraphPad Prism 6.0 software (La Jolla, USA) was used for statistical analysis. All the data were expressed as mean ± SD from at least three independent experiments.

RESULTS

Establishing the CMT-N7 cell line from a canine mammary tumor: Over twenty primary cultures were derived from female dogs with CMT admitted to Nanjing Agricultural University's Veterinary Hospital. One culture, CMT-N7, was successfully established and passaged >120 times. As shown in Fig. 1A, H&E staining of CMT samples revealed ducts and adenoid structures with disrupted columnar cells spanning 3-5 layers, multi-layered oval cells with pleomorphism, mitotic activity, and hyperchromatic nucleoli.

Morphology of CMT-N7 cells: As shown in Fig. 1B, CMT-N7 cells showed multilayered epithelioid morphology with atypia: large, irregular nuclei and multiple nucleoli. Throughout the passage, there were no significant alterations in cell morphology, indicating that the growth of CMT-N7 remained stable and homogeneous. TEM analysis further revealed their epithelioid morphology with large irregular nuclei and cytoplasm filled with organelles like mitochondria, ribosomes, endoplasmic reticulum, intermediate filaments, and vacuoles (Fig. 1C).

Growth study and doubling time of CMT-N7 cells: Cell growth was continuously monitored, and the resulting growth curve was depicted in Fig. 2A. The observed "S-shaped" cell growth curve indicated distinct phases of slow growth incubation, logarithmic proliferation, and plateau stability. The doubling time of CMT-N7 cells was 20.34 h.

Karyotype analysis of CMT-N7 cells: The CMT-N7 cells exhibited both numerical and structural abnormalities in their chromosomes as determined by karyotype analysis, with the majority of cells being aneuploid. As depicted in Fig. 2B, the chromosome counts in the CMT-N7 cell line ranged from 70 to 90, whereas the normal count for canines was typically 78. Furthermore, a fusion event occurred between two monocentric chromosomes, resulting in the formation of metacentric chromosomes with two arms.

Mycoplasma detection of CMT-N7 cells: As shown in Fig. 2C, CMT-N7 cells were tested for the presence of mycoplasma by a PCR method and found to be free of mycoplasma contamination.

The metastasis ability of CMT-N7 cells *in vitro*: To evaluate CMT-N7 invasion and mobility capability, wound healing assay and transwell assay were performed.

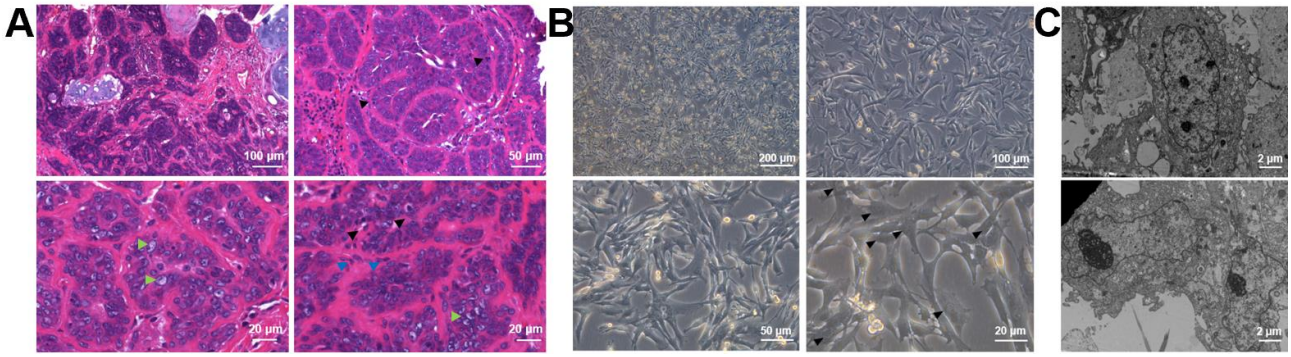

Fig. 1: Characterization of primary canine mammary tumor tissues and its cell line CMT-N7. (A) Hematoxylin and eosin (H&E) staining of tumor tissues revealed predominantly duct and adenoid structures. The cells displayed a disorganized arrangement, size variation, significant cellular atypia (green arrow), prominent mitotic activity (blue arrow), and deep staining of the nucleoli (black arrow). Scale bar = 20 μm. (B) Optical microscopy of CMT-N7 cells showed multilayer growth and epithelioid or spindle-shaped cell morphology. The cells exhibited obvious atypia, with large and irregular nuclei and multiple nucleoli (black arrow). Scale bar = 20 μm. (C) Ultrastructural images obtained via transmission electron microscopy (TEM) revealed that CMT-N7 cells contained a variety of organelles and some vacuolar structures. Scale bar = 2 μm.

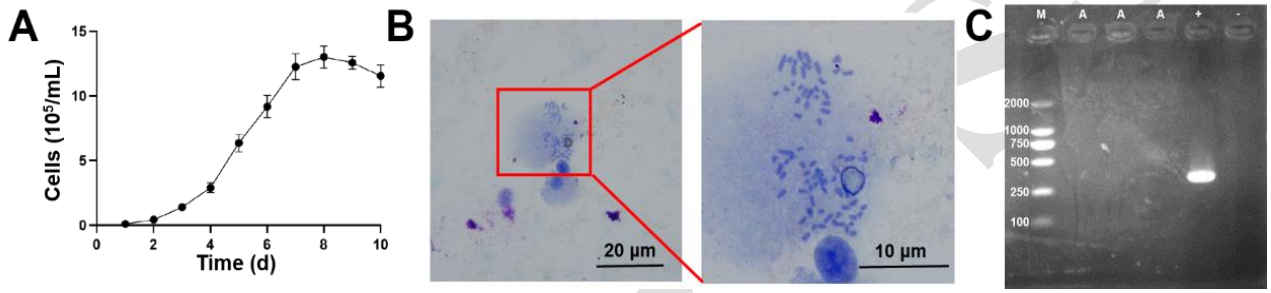

Fig. 2: Growth curve, karyotype analysis, and mycoplasma detection of the CMT-N7 cell line. (A) The growth curve of CMT-N7 cell line. (B) The number of chromosomes in CMT-N7 cell line was between 70 and 90. (C) Mycoplasma detection of the CMT-N7 cell line exhibited no mycoplasma contamination. M, maker; A, CMT-N7 cells; +, positive control; –, negative control.

As shown in Fig. 3A, 24 h after scratching, newly grown CMT-N7 cells migrated to the middle area and covered the scratch area. Meanwhile, the CMT-N7 cells showed strong invasion ability through the basement membrane (Fig. 3B).

Characterization of CMT-N7 cells: The cellular expression characteristics of CMT-N7 cells were depicted in Fig. 4, indicating that cells showed positive staining for ER, α-SMA, CK-14, SOX-2, Vimentin, Ki-67, E-cadherin and COX-2. Notably, CMT-N7 cells exhibited co-expression of both epithelial and mesenchymal markers.

Tumorigenesis potential of CMT-N7 cells in nude mice: The cell suspension of CMT-N7 was subcutaneously injected into 10 female BALB/c nude mice, resulting in tumor formation observed in all the mice after 2 weeks of inoculation. Additionally, significant angiogenesis was observed on the tumor surface (Fig. 5A). Tumor volumes were measured and tumor growth curves were plotted weekly (Fig. 5B). In addition, the tumor cells exhibited marked atypia, increased nucleoli, and a high mitotic index (Fig. 5C).

Immunohistochemical analysis of xenograft tumors: Immunohistochemical analysis was performed to compare the expression levels of ER, PR, HER-2, Ki-67, E-cadherin, Vimentin, and COX-2 in CMT-N7 xenografts. As depicted in Fig. 6, the results revealed negative expression of HER-2 and PR, while positive expression of ER, Ki-67, E-cadherin, Vimentin, and COX-2 was observed (Fig. 6).

DISCUSSION

As the lifespan of human and companion animals continues to increase, the incidence of cancer also rises. Among companion animals, dogs are one of the most commonly affected species and exhibit a high occurrence of spontaneous CMT, particularly in older, unspayed female dogs (Yeom *et al*., 2023). Numerous studies have demonstrated that mammary tumors account for approximately 50% of all tumors in female dogs, contributing to 82% of reproductive-related diseases in this population (Luu *et al*., 2019; Reis *et al*., 2020; Pieczewska *et al*., 2021). CMT and HBC share notable similarities in terms of epidemiological, histological, and clinical features (Abdelmegeed and Mohammed, 2018; Nam *et al*., 2020). In addition, the average lifespan of dogs is shorter than that of humans and the number of oestrus is higher, so spontaneous canine mammary tumors occur and develop more rapidly than those in humans. Therapeutic research on canine mammary cancer is not only conducive to improving the clinical cure rate of canine mammary cancer and prolonging the life span of dogs with the disease but also helpful to the study of human mammary cancer and the development of new anti-tumor drugs. The use of cell lines for the detection of cell growth and progression in cancer research offers multiple advantages such as standardization and

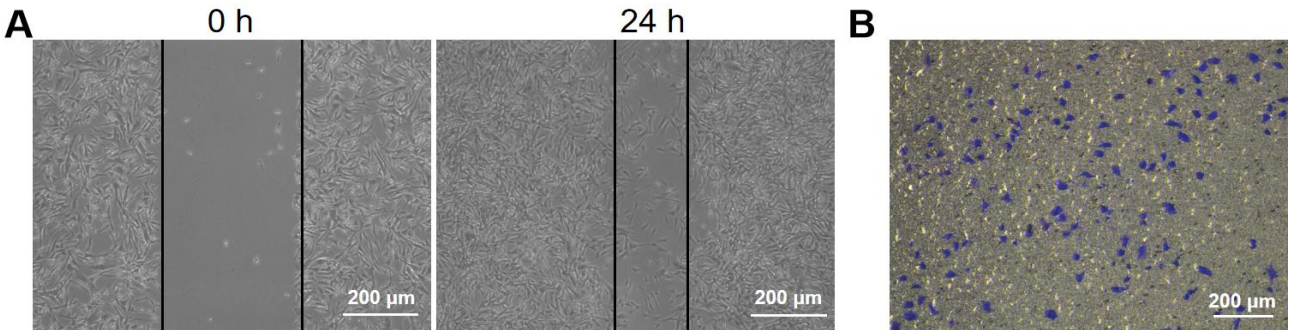

Fig. 3: Migration and invasion ability of CMT-N7 cells were assessed. (A) Representative images depicting the characteristic process of wound healing in CMT-N7 cells at 0 and 24 h were presented. The black vertical lines in the images defined the open wound areas. Scale bar = 200 µm. (B) Typical photographs of CMT-N7 cells' invasion were taken under a microscope. Staining was performed using crystal violet for 10 min at room temperature, followed by washing with PBS. Scale bar = 200 µm.

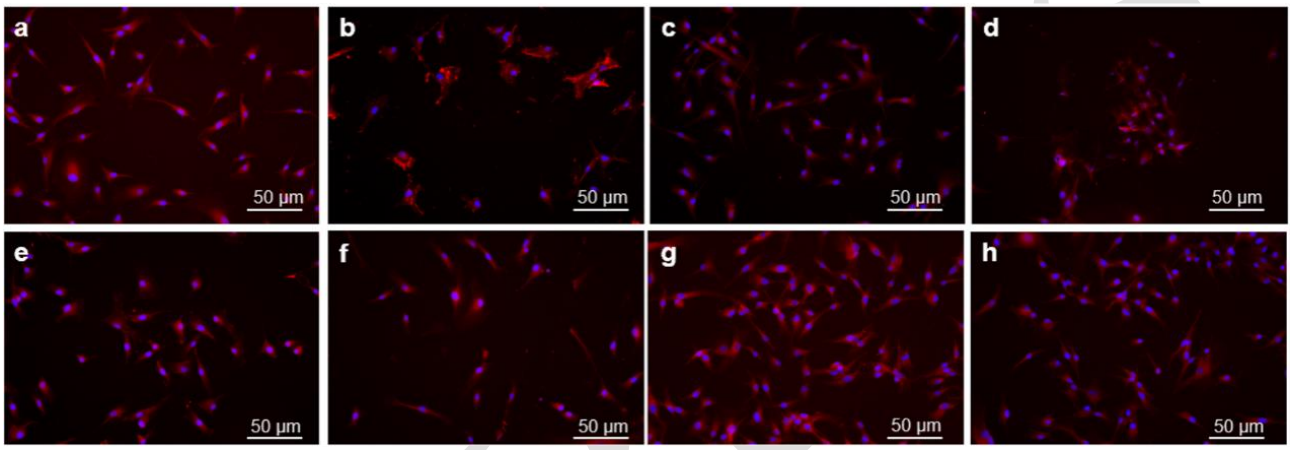

Fig. 4: Indirect immunofluorescence assay characteristics of CMT-N7 cells. (a) ER showed weak positivity, suggesting low expression levels. (b) α-SMA also exhibited weak positivity, indicating minimal smooth muscle cell differentiation. (c) CK-14 demonstrated weak positivity, reflecting a basal cell phenotype. (d) SOX-2 showed weak positivity, indicating low levels of this transcription factor. In contrast, (e) Vimentin, (f) Ki-67, (g) E-cadherin, and (h) COX-2 all exhibited strong positivity, indicating higher levels of expression of these proteins. The immunofluorescence labeling was performed using FITC and DAPI. Scale bar = 50 µm.

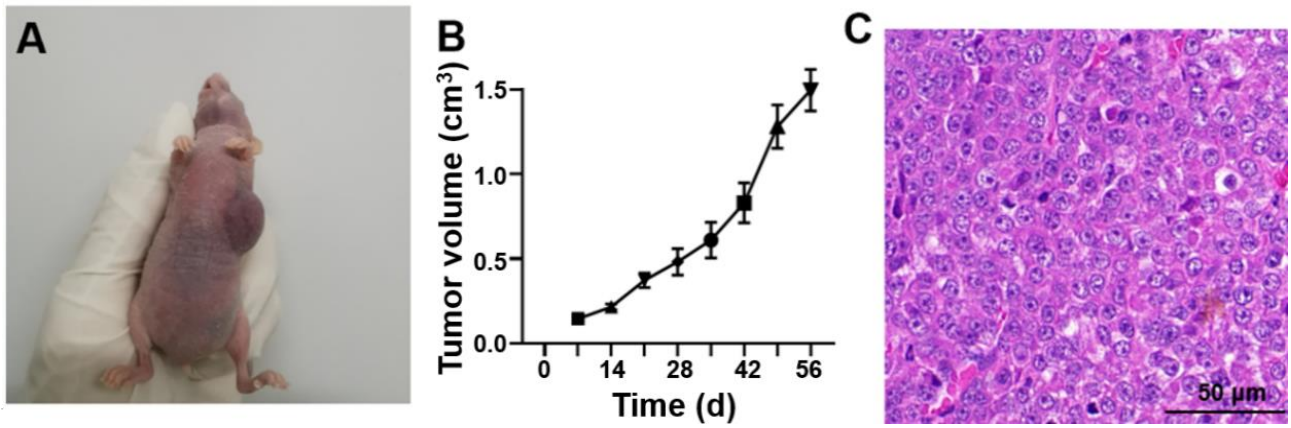

Fig. 5: Tumorigenicity assay in nude mice. (A) Picture of CMT-N7 cells xenograft nude mice. (B) Tumor growth curve in the nude mice. (C) H&E staining of xenograft CMT-N7 cells.

reproducibility, efficiency and affordability, model applicability, technological versatility and innovation, as well as in-depth analysis and mechanistic studies. CMT can serve as a valuable model for researching HBC (Pinho *et al*., 2012; Mei *et al*., 2021). The utilization of cell lines in cancer research has demonstrated numerous advantages in detecting cellular growth and progression. In particular, the establishment of canine mammary tumor cell lines plays a crucial role in enhancing our understanding of the development of canine mammary tumors, thereby providing a novel cell model for studying HBC.

Cell isolation and purification are key to the difficulty of primary culture. However, some malignant cells can be naturally purified to generate cell lines capable of unlimited proliferation *in vitro*, and established breast cancer cell lines are widely used in a variety of studies, particularly as *in vitro* models of cancer (Burdall *et al*., 2003). In the current study, CMT-N7 cells exhibited

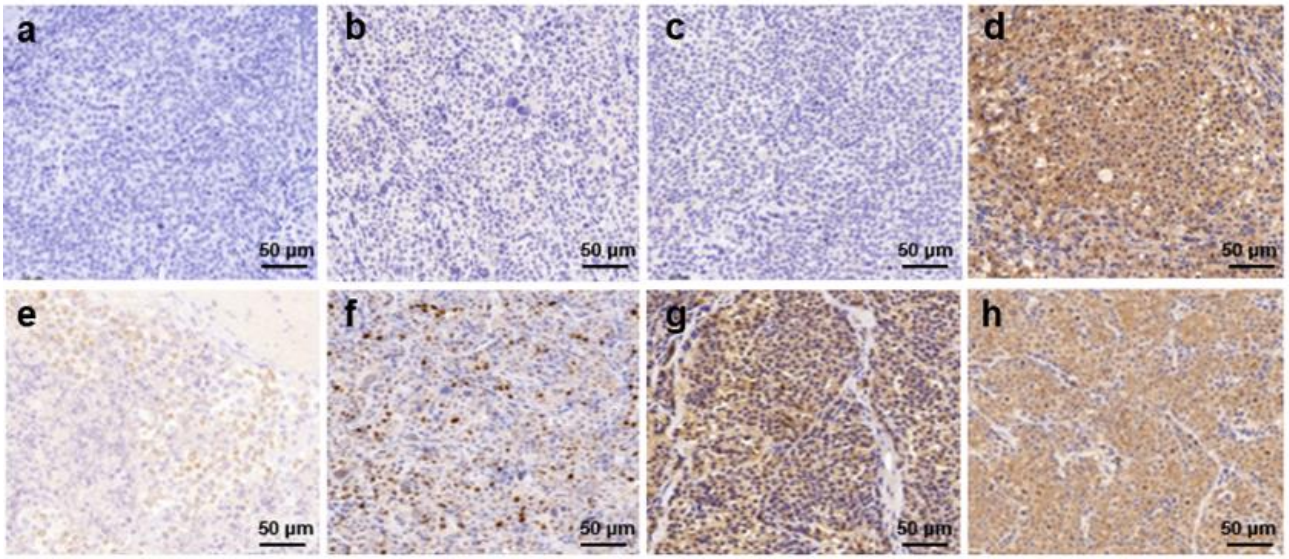

Fig. 6: Immunohistochemical features of xenograft tumors. (a) Negative control showed no staining, confirming the specificity of the assay. (b) HER-2 and (c) PR were negative in tumor tissue, suggesting minimal or no expression. In contrast, (d) ER, (e) Ki-67, (f) E-cadherin, (g) Vimentin, and (h) COX-2 showed positive staining, indicating their presence. Scale bar = 50 µm.

epithelioid morphology and demonstrated multi-layered growth. Notably, the contact inhibition of CMT-N7 cells exhibited a reduction, and the doubling time was shorter 20.34 h, indicating that the cell had a strong proliferation ability. Furthermore, the established cell line demonstrated continuous growth even after cryopreservation, suggesting its long-term viability. The CMT-N7 cells had undergone stable passages for over 120 generations and had been cultivated for more than a year. With these characteristics, CMT-N7 had the potential to serve as a valuable cellular model for investigating CMT. Each line possesses unique characteristics, making it important to continuously cultivate new cell lines. At present. although numerous pre-existing breast cancer cell lines have been identified in both humans and dogs (Lehmann *et al*., 2011; Raposo *et al*., 2017), each line possesses unique characteristics, making it crucial to continuously cultivate new cell lines to enrich the variety and quantity of available lines, thereby laying a foundation for the development of translational medicine. The CMT- N7 cell line adds to existing breast cancer cell lines and provides researchers with new tools to study CMT.

In vitro, cultured tumor cells must undergo a series of biological identification before conducting subsequent tests such as cancer pathogenesis and anti-cancer drug screening. Therefore, the present study aimed to characterize the morphology, growth kinetics, karyotype analysis, migratory potential, tumorigenicity, and associated biomarkers of CMT-N7 cells. These comprehensive evaluations are essential for establishing a reliable and representative model for further research and clinical applications. The results demonstrated that CMT-N7 cells exhibited a multilayered growth pattern, following an "S" shaped growth curve consistent with the characteristic behavior of tumor cells. Microscopic and ultrastructural analysis revealed the epithelioid morphology of the cells, characterized by abundant nucleoli and cytoplasm. Chromosome analysis revealed common variations in chromosome number and structure consistent with the characteristics of tumor cell chromosomal changes. However, the precise structural alterations of individual chromosomes were not specifically investigated in this study. The wound healing assay demonstrated that CMT-N7 cells exhibited robust migratory capacity even under conditions of serum deprivation. Subcutaneous injection of the cell suspension into the left breast pad of BALB/c nude mice successfully initiated tumor growth, accompanied by notable angiogenesis on the tumor surface. The histopathological analysis further revealed the presence of tumor cells displaying distinct dark nucleoli and prominent mitotic figures, indicative of active cell proliferation. Compared with other established canine mammary cancer cell lines, the CMT-N7 cell line has the same "S"-shaped growth curve and epithelioid morphology, and possesses strong migration potential, and tumorigenicity consistent with tumor aggressiveness (Mei *et al*., 2021; Chen *et al*., 2023). In the future, by comparing CMT-N7 with various breast tumor models, the biological properties of CMT-N7 will be further clarified, and the prospects for its scientific research and clinical application will be expanded.

Immunohistochemical analysis of xenograft tumors and CMT-N7 was positive for ER, CK-14, and α-SMA, but negative for HER-2 and PR. ER, PR, HER-2 and Ki-67 are markers for breast cancer subtypes (Barnard *et al*., 2015; Chen *et al*., 2023). Approximately 15-20% of breast cancers are ER/PR negative (Cavaco *et al*., 2021). HER-2 is negative/low in 75-85% of cases (Yang *et al*., 2021). Ki-67, a proliferation marker, predicts poor prognosis in breast cancer (Pena *et al*., 2003; Kaufmann *et al*., 2020). High Ki-67 expression indicates faster proliferation and malignancy (Gama *et al*., 2008b; Bonacho *et al*., 2020). CK-14 and α-SMA positivity indicates epithelial origin. CK-14 expression varies during cell differentiation and in different epithelial cells (Bragulla and Homberger, 2009). CK-14 is differentially expressed in malignancies such as lung small cell carcinoma, breast cancer (Cheung *et al*., 2013), and esophageal cancer, which is important for tumor diagnosis and prognosis. CMT-N7 cells showed

positive expression of vimentin, E-cadherin, SOX-2, and COX-2. Vimentin regulates cytoskeletal architecture and cell force in EMT, which is associated with metastatic spread (Liu *et al*., 2015; Kim *et al*., 2023). E-cadherin expression correlates with adverse outcomes in primary tumors (Roussos *et al*., 2010; Mendonsa *et al*., 2018; Kaszak *et al*., 2020). These markers help to identify EMT processes. Chronic inflammation drives tumorigenesis via cytokines and chemokines released by inflammatory cells, which promote angiogenesis and tumor growth (Carvalho *et al*., 2016). COX-2, which is undetectable in normal tissues, is induced by inflammatory responses, growth factors, tumor promoters, and oncogenes. In humans, COX-2 overexpression correlates with recurrence, metastasis, and prognosis in HBC and other cancers (Che *et al*., 2022). In veterinary medicine, COX-2 expression is higher in malignant CMT than in benign CMT (Queiroga *et al*., 2010). SOX-2, a key transcription factor in embryonic development and stemness maintenance, affects cancer progression by influencing EMT, proliferation, and cancer stem cell formation (Avilion *et al*., 2003; Novak *et al*., 2020). The CMT-N7 cell line is classified as a luminal B subtype of canine mammary tumor cells and may undergo epithelial-mesenchymal transition. Therefore, this cell line serves as a valuable in vitro model to study the mechanism underlying EMT in canine breast cancer.

Conclusion: A novel canine mammary gland cancer cell line, CMT-N7, has been successfully established and thoroughly characterized. This cell line holds tremendous research significance for future studies focused on understanding the underlying mechanism of tumorigenesis and progression in both HBC and CMT. In particular, the CMT-N7 cell line provides a valuable resource for investigating the EMT mechanism within mammary cancer cell lines. By utilizing this cell line, researchers can gain insights into the molecular processes involved in cancer progression and metastasis, ultimately paving the way for the development of innovative therapeutic strategies. The similarities between CMT-N7 and human breast cancer cells further strengthen its relevance as a valuable translational research model aiming at benefiting both human and veterinary patients.

Authors contribution: Xinhao Song: Formal analysis, Conceptualization, Methodology, Data curation, Writing-original draft. Shasha Gao: Methodology, Investigation, Formal analysis. Shiheng Hu: Formal analysis, Data curation. Tian Fang: Methodology, Data curation. Xiaolin Xu: Methodology, Formal analysis. Xin Lv: Methodology, Validation. Xiuge Gao: Software, Formal analysis. Mengjuan Lin: Software, Investigation. Lin Peng: Methodology. Meng Li: Software. Yingjun Lv: Software. Shanxiang Jiang: Conceptualization, Methodology. Dawei Guo: Conceptualization, Methodology, Project administration, Writing-review & editing, Funding acquisition.

Declaration of Competing Interest: The authors declare no conflicts of interest.

Acknowledgments: This work was supported by grants from the Fundamental Research Funds for the Central Universities (KYCXJC2024002&YDZX2023006) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

REFERENCES

Abdelmegeed S M and Mohammed S, 2018. Canine mammary tumors as a model for human disease. Oncol Lett 15: 8195-8205.

Avilion A A, Nicolis S K, Pevny L H, et al., 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Gene Dev 17: 126-140.

Barnard M E, Boeke C E and Tamimi R M, 2015. Established breast cancer risk factors and risk of intrinsic tumor subtypes. Acta Bioch Bioph Sin 1856: 73-85.

Bonacho T, Rodrigues F and Liberal J, 2020. Immunohistochemistry for diagnosis and prognosis of breast cancer: a review. Biotech Histochem 95: 71-91.

Bragulla H H and Homberger D G, 2009. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. J Anat 214: 516-559.

Burdall S S, Carvalho S, Cabral J, et al., 2012. Canine tumors: a spontaneous animal model of human carcinogenesis. Transl Res 159: 165-172.

Carvalho M I, Silva-Carvalho R, Pires I, et al., 2016. A comparative approach of tumor-associated inflammation in mammary cancer between humans and dogs. Biomed Res Int 2016: 4917387.

Cavaco M, Fraga P, Valle J, et al., 2021. Development of breast cancer spheroids to evaluate cytotoxic response to an anticancer peptide. Pharmaceutics 13: 1863.

Che L, Wu JS, Du ZB, et al., 2022. Targeting mitochondrial cox-2 enhances chemosensitivity via drp1-dependent remodeling of mitochondrial dynamics in hepatocellular carcinoma. Cancers 14: 821.

Chen A, Ye S, Zheng J, et al., 2023. Establishment and characterization of a HER2-enriched canine mammary cancerous myoepithelial cell line. BMC Vet Res 19: 22.

Cheung K J, Gabrielson E, Werb Z, et al., 2013. Collective invasion in breast cancer requires a conserved basal epithelial program. Cell 155: 1639-1651.

De la Roca-Chiapas JM, Barbosa-Sabanero G, Martinez-Garcia JA, et al., 2016. Impact of stress and levels of corticosterone on the development of breast cancer in rats. Psychol Res Behav Ma 9: 1-6.

Esparza-López J, Alvarado-Muñoz JF, Escobar-Arriaga E, et al., 2019. Metformin reverses mesenchymal phenotype of primary breast cancer cells through STAT3/NF-κB pathways. BMC Cancer 19: 728.

Gama A, Paredes J, Gartner F, et al., 2008. Expression of E-cadherin, P-cadherin and beta-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. Vet J 177: 45-53.

Kaszak I, Witkowska-Piłaszewicz O, Niewiadomska Z, et al., 2020. Role of cadherins in cancer-a review. Int J Mol Sci 21: 7624.

Kaufmann C, Kempf W, Mangana J, et al., 2020. The role of cyclin D1 and Ki-67 in the development and prognostication of thin melanoma. Histopathology 77: 460-470.

Kim J H, Park S, Jung E, et al., 2023. A dual-action niclosamide-based prodrug that targets cancer stem cells and inhibits TNBC metastasis. Proc Natl Acad Sci U S A 120: e2304081120.

Lehmann B D, Bauer J A, Chen X, et al., 2011. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 121: 2750-2767.

Lin M, Song X, Zuo R, et al., 2023. Nano-encapsulation of halofuginone hydrobromide enhances anticoccidial activity against Eimeria tenella in chickens. Biomater Sci 11(5): 1725-1738.

Lindblad-Toh K, Wade C M, Mikkelsen T S, et al., 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature 438: 803-819.

Liu C Y, Lin H H, Tang M J, et al., 2015. Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget 6: 15966-15983.

Luu S, Bell C, Schneider S, et al., 2019. A. Connexin 26 and connexin 43 in canine mammary carcinoma. Vet Sci 6(4): 101.

Markkanen E, 2019. Know Thy Model: Charting molecular homology in stromal reprogramming between canine and human mammary tumors. Front Cell Dev Biol 7: 348.

Mei C, Xin L, Liu Y, et al., 2021. Establishment of a new cell line of canine mammary tumor CMT-1026. Front Vet Sci 8: 744032.

Mendonsa A M, Na T Y and Gumbiner B M., 2018. E-cadherin in contact inhibition and cancer. Oncogene 37: 4769-4780.

Novak D, Huser L, Elton J J, et al., 2020. SOX2 in development and cancer biology. Semin Cancer Biol 67: 74-82.

Ou G, Jiang X, Gao A, et al., 2021. Celastrol inhibits canine mammary tumor cells by inducing apoptosis via the caspase pathway. Front Vet Sci 8: 801407.

Pena L, Perez-Alenza M D, Rodriguez-Bertos A, et al., 2003. Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases. Breast Cancer Res Tr 78: 141-148.

Pieczewska B, Glinska-Suchocka K, Nizanski W, et al., 2021. Decreased size of mammary tumors caused by preoperative treatment with aglepristone in female domestic dogs (canis familiaris) do not influence the density of the benign neoplastic tissue measured using shear wave elastography technique. Animals 11: 527.

Queiroga F L, Pires I, Lobo L, et al., 2010. The role of Cox-2 expression in the prognosis of dogs with malignant mammary tumours. Res Vet Sci 88: 441-445.

Raposo T P, Arias-Pulido H, Chaher N, et al., 2017. Comparative aspects of canine and human inflammatory breast cancer. Semin Oncol 44: 288-300.

Reis LA, Garcia APV, Gomes EFA, et al., 2020. Canine mammary cancer diagnosis from quantitative properties of nonlinear optical images. Biomed Opt Express 11: 6413-6427.

Rivera P and von Euler H, 2011. Molecular biological aspects on canine and human mammary tumors. Vet Pathol 48: 132-146.

Roussos E T, Keckesova Z, Haley J D, et al., 2010. AACR special conference on epithelial-mesenchymal transition and cancer progression and treatment. Cancer Res 70: 7360-7364.

Song X, Lin M, Fang T, et al., 2023. Maduramicin-guided nanotherapy: A polymeric micelles for targeted drug delivery in canine mammary tumors. Biomed Pharmacother 170: 116062.

Srisawat W, Nambooppha B, Pringproa K, et al., 2020. A preliminary study of the cross-reactivity of canine mage-a with hominid monoclonal antibody 6c1 in canine mammary gland tumors: an attractive target for cancer diagnostic, prognostic and immunotherapeutic development in dogs. Vet Sci 7(3): 109.

Yang L, Li Y, Bhattacharya A, et al., 2021. Loss of peptidase D binding restores the tumor suppressor functions of oncogenic p53 mutants. Commun Biol 4: 1373.

Yeom J, Cho Y, Ahn S, et al., 2023. Anticancer effects of alpelisib on PIK3CA-mutated canine mammary tumor cell lines. Front Vet Sci 10: 1279535.

Zhang H, Pei S, Zhou B, et al., 2018. Establishment and characterization of a new triple-negative canine mammary cancer cell line. Tissue Cell 54: 10-19.