Establishment and Characterization of An HER2-enriched Canine Mammary Cancerous Myoepithelial Cell Line with Stem Cell Properties

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Abstract

Background

Canine mammary tumors (CMTs) have a poor prognosis, along with tumor recurrence and metastasis. Cell lines and organoids are vital *in vitro* models for CMT research. Although anti-HER2 therapy improves the patients' outcome, anti-HER2 drug resistance is still a big challenge and yet no canine HER2-enriched cell line has been established. This study aimed at establishing such a novel canine mammary cell line. CMT-1 cell line was obtained from clinical tumor CMT-1 and characterized molecularly through qPCR, western blotting, immunochemistry, immunofluorescence. Its doubling time, migration rate and stemness were evaluated using growth study, wound healing assay and 3D cultures respectively. To determine its tumorigenesis, xenograft transplantation was performed.

Results

CMT-1 is a mixed canine mammary carcinoma that stains negative for estrogen receptors (ER) and progesterone receptors (PR), but positive for human epidermal growth receptor-2 (HER2), defined as HER2-enriched subtype. In this study, a CMT-1 cell line obtained from CMT-1 tumor was confirmed as myoepithelial cells and was successfully cultured for more than 50 passages showing the same immunoreactivity to ER, PR, and HER2. The doubling time of CMT-1 cell line was 26.67 h. A spontaneous epithelial to mesenchymal transition (EMT) was noticed during cell cultures. EMT-induced CMT-1 cells showed a faster migration rate compared to the original CMT-1 cells, but no significance was detected. CMT-1 cells grew on a 3D culture and formed grape-like, solid, and cystic mammosphere at different stages, indicating CMT-1 cell line has stem cell property. Inoculation of CMT-1 cells induced a mixed HER2-enriched mammary tumor with metastasis in mice.

Conclusions

A canine cancerous HER2-enriched myoepithelial cell line was successfully established and the first canine mammosphere was documented in this study. We are expecting this novel cell line and mammosphere could be used as a model for CMT tumorigenesis and new anti-HER2 drug screening in the future.

1 Background

Canine mammary tumors (CMT) are the most commonly seen neoplasm in intact female dogs, with approximately 50% being malignant [1]. Currently, the classification of CMT is based on tumor characteristics, such as morphology, tumor cell origins, and molecular markers [2–4]. However, subtle changes in mammary structure increase the difficulty of diagnosing CMT subtypes. To better identify tumor subtypes and obtain a more accurate diagnosis, immunohistochemistry (IHC) has gained

popularity [4–6]. In comparative medicine, veterinary researchers have applied the immunophenotypes adopted from human breast cancer (HBC) to CMT using protein biomarkers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Based on the expression of the relevant biomarkers, the subtypes are classified as luminal A, luminal B, HER2-enriched, triple-negative, and basal-like [7]. The relationship between CMT immunophenotypes and prognostic significance has been extensively studied [5, 8, 9]

The *HER2* gene belongs to the epidermal growth factor receptor (EGFR) family, activation of which is associated with increased proliferation, angiogenesis, invasiveness, and epithelial-mesenchymal transition (EMT) [10, 11]. Approximately 15–20% of the HBC is classified as HER2 positive with HER2-enriched being the most common subgroup, which is associated with a worse prognosis [12]. On the other hand, the prevalence of HER2-positive CMT is 8.3–22% [9, 13], similar to that of HBC. With the application of trastuzumab, an anti-HER2 antibody, the overall survival improved significantly for HER2 positive patients [9]. However, metastatic HER2 positive HBC remains deadly owing to primary or acquired resistance to anti-HER2 therapies [14, 15]. Nevertheless, only a few HER2-enriched cell lines have been developed [16].

The mammary gland is composed of an epithelial cell lineage and a myoepithelial (ME) cell lineage. ME cells are contractile cells that express α -smooth muscle actin (α -SMA) and are arranged in a continuous monolayer in the mammary duct and appear in a stellate shape around the mammary acini [17]. Recently, ME cells have captured some attention owing to their potential tumor-suppressive effect [18]. To the best of our knowledge, neither canine HER2-enriched nor ME cell lines have been reported.

It is widely believed that tumor recurrence or therapy resistance are related to cancer stem cells (CSCs) [19]. CSCs are found in tumors that could generate malignant tumors through self-renewal and differentiation. CSCs play a crucial role in the EMT process, which has been shown to be related to cancer metastasis, recurrence, and resistance [20]. It is believed that cells at different stages of EMT exhibit different degrees of stemness [21]. With their differentiation abilities, stem cells are able to grow on 3D culture to form an organoid model that mimics *in vivo* organs. Considering the complexity of the biological behavior of mammary tumors, 3D cultures and breast cancer organoids outweigh 2D culture for the study of cellular, structural, and microenvironmental features [22, 23]. However, no CMT organoid has been reported.

In summary, our study showed a successful establishment of a HER2-enriched canine mammary cell line and examined its morphology, growth pattern, protein expression, and stemness. This study also explored the potential of a CMT organoid model developed for veterinary science.

2 Results

2.1 Establishment of the CMT-1 cell line from a malignant HER2-enriched CMT

The primary cell culture was obtained from a 10-year-old female intact Chihuahua that was presented to the South China Agricultural University Veterinary Teaching Hospital for a mammary mass. The canine mammary tumor was diagnosed based on its signalment, history, and clinical findings. No metastasis was noted on the thoracic radiographs and abdominal ultrasound. Standard mastectomy was performed after informed consent from the owner. Histopathology revealed that mammary epithelial cells formed small tubular structures and neoplastic myoepithelial cells were spindoloid and located in the extracellular matrix. There was moderate anisocytosis and anisokaryosis. Rare mitotic figures were seen (Fig. 1A-B). This is consistent with mixed low grade canine mammary carcinoma. IHC detected no expression of ER and PR, moderate expression of HER2, and strong expression of E-cadherin (Fig. 1C-G). According to the Herceptest™ interpretation manual, CMT-1 was categorized as HER2-enriched (2+) subtype.

2.2 Growth studies and doubling time of the CMT-1 cell line

In order to investigate the growth property and morphology of the CMT-1 cell line, we observed the shape of CMT-1 during cultivation passage and performed a growth study assay. CMT-1 cells were polygonal cells with, initially, multiple nuclei in one cell (passage 9). With increasing cell passage number, the CMT-1 cells became less spindoloid and tended to be uniformly round to oval shape at passage 50 (Fig. 2A-B). This may indicate that CMT-1 became more stable in characteristics allowing for further study. Growth studies showed that the doubling time of the cell line was 26.67 h (Fig. 2C).

2.3 CMT-1 cells were HER2-enriched myoepithelium

To identify which subgroup CMT-1 cells belonged to, we detected the expression of ER, PR, and HER2 using WB and IFA. The expression of E-cadherin, vimentin, and α -SMA was also detected by IFA to determine the cell lineage of CMT-1 cells. Morphologically, CMT-1 cells were spindle-shaped under a light microscope (Fig. 2A). Molecularly, CMT-1 cells (passage 50) were negative for ER and PR but positive for HER2 in WB assays (Fig. 3). MDCK cells were used as a positive control for ER and negative control for PR and HER2. Compared to MDCK cells, the CMT-1 cell line was negative for ER and PR but positive for HER2. Interestingly, as the CMT-1 cell passage increased, the CMT-1 cell line lost ER expression but gained HER2 expression by passage 50. Uyama et al. confirmed that CHMp cells, another canine mammary tumor cell line, were positive for E-cadherin using flow cytometry but their hormonal receptors and HER2 status have not been evaluated [24]. In this study, we detected moderate ER expression, weak PR expression, and no HER2 expression of CHMp cells. Hence, CHMp cells were categorized as a luminal subtype. In IF images, although a scant amount of ER and PR signaling was received, given the location of the signals, CMT-1 cells were considered ER and PR negative [25]. Meanwhile, CMT-1 cells showed a moderate to strong signal of HER2. Moreover, the CMT-1 cell line demonstrated weak reaction to E-cadherin but a moderate to strong reaction to vimentin and α -SMA in the cytoplasm and cytomembrane, respectively (Fig. 4). Therefore, we defined CMT-1 cells as HER2-enriched myoepithelial cells.

2.4 Epithelial-mesenchymal transition property and stemness of the CMT-1 cells

Our *in vivo* study showed that CMT-1 cells could induce metastasis; however, no metastasis was detected in the original patient at 14 months post-surgery. In order to assess the abilities of migration rate between cell passages, CMT-1 cells (passage 5 and passage 50) were used for wound healing assays. Despite no notable difference in migration distance over 48 h between the two cell passages, an obvious shorter open wound distance was noted in CMT-1 cells (passage 50), indicating that it may exhibit mildly increased migration abilities during long term cultivation passage (Fig. 5). The change in migration rate was likely related to EMT. Therefore, the expression of E-cadherin and vimentin was tested using WB with four different cells. MDCK and CHMp cells were used as the control. CMT-1 cells (passage 5) expressed the highest level of E-cadherin among the four different cells. However, CMT-1 cells (passage 50) showed low expression of E-cadherin, which was consistent with the previous IFA results. Overtime, CMT-1 cells (passage 50) reduced E-cadherin expression and gained more vimentin expression (Fig. 6A). At the same time, the mRNA expression level of E-cad and vimentin in CMT-1 cells (passage 5 and passage 50) were evaluated using qPCR, which showed a statistical significance between passages (Fig. 6B). This suggested that CMT-1 cells underwent spontaneous EMT leading to increased migration rate.

Some cancer cells may obtain stemness after gene mutation. To test the potential pluripotent stemness of CMT-1 cells, CMT-1 was placed in matrigel for 3D culture. Organoid developed from CMT-1 single cells demonstrated grape-like, solid, cystic, or mixed morphologies (Fig. 7). During organoid culture, the morphology of the organoid became more solid and cystic. This suggested that the CMT-1 cell line should be stem-cell like and able to differentiate, and be developed into a mammosphere.

2.5 Tumorigenicity of CMT-1 cells in mice

In order to evaluate the tumorigenicity of this cell line, CMT-1 cells were injected subcutaneously into the left inguinal mammary fat pad of mice, with the injection bleb being completely absorbed within 1 min. The tumor grew slowly in the early stages. Most of the tumors appeared as small bean-like soft solid masses (Fig. 8A). The tumor size varied in mice but the overall size increased throughout the experiment (Fig. 8B). Surprisingly, diaphragmatic metastatic lesions were noticed during necropsy in three mice (Fig. 8A). Seven mice were confirmed to have mixed mammary adenocarcinoma with no metastasis histologically, whereas three mice were consistent with mixed mammary adenocarcinoma with metastatic diaphragmatic adenocarcinoma. The epithelial cells formed tubular structures. The cells had slightly vacuolar to granular cytoplasm with the nuclei being round to oval with vesicular chromatin (Fig. 8C). There was moderate anisocytosis and anisokaryosis. The skeletal muscle of the diaphragm showed degeneration and atrophy with infiltrates of neoplastic cells (Fig. 8D). The immunophenotype markers of the xenograft tumor were similar to those of the canine primary tumor, which was ER and PR negative but HER-2, α -SMA, and E-cadherin positive. (Fig. 9). Interestingly, although the immunophenotype markers of the metastatic lesion were similar to the primary xenograft tumor, it appeared to have stronger HER2 and α -SMA staining. Therefore, we suspected the metastatic tissue was mainly composed of myoepithelial cells. This *in vivo* experiment demonstrated that the CMT-1 cell line could produce tumors that resembled the original tumor but with greater malignancy both clinically and molecularly.

3 Discussion

3.1 Clinical significance of HER2 and myoepithelium

HER2/ErbB2 is an oncogenic gene associated with tyrosine kinase, facilitating cell proliferation, disruption of cell adhesion and cell polarity, invasiveness, and metastasis [26]. Among all the subtypes of HBC, HER2-positive breast cancer accounted for 11–30% with the HER2-enriched being the most representative subgroup [27]. HER2-enriched subtype tended to metastasize [28]. Therefore, HER2-enriched cell lines are an essential *in vitro* model for the study of its biological behaviors and to evaluate potential new regimens. Indeed, several HER2 enriched cell lines have been developed such as HER2BT-20 and SK-BR3[16]. Similarly, CMT was subdivided based on its molecular expressions and each subtype has its own clinical significance that is worth studying. Over the last decade, several cell lines have been established to study CMT including CMT-7364, CMT-7367/5-FU, and B-CMT (triple negative), FR37-CMT (ER⁺, HER2⁺), and IPC-366 (basal-like) [29–33]. Nonetheless, a HER2-enriched CMT cell line has not been reported. In the current study, CMT-1 cells expressed no ER and PR, but did express HER2 in WB, IFA, and IHC. Therefore, we successfully established a canine HER2-enriched mammary cell line.

The mammary gland consists of luminal epithelial cell lineage and basal ME cell lineage. ME cells are contractile epithelial cells that lie in between the luminal epithelial cells and basement membrane [18, 34]. In addition to cell morphology, the use of cell markers is another way to identify myoepithelium. α -SMA is a ME cell marker of choice in dogs. Other ME cell markers include cytokeratins, calponin, and protein p63 [35]. In the present study, ME cells were successfully isolated from the clinical case and identified based on morphology and molecular expression.

In our study, a 14-month follow-up with the patient confirmed no CMT recurrence after routine mastectomy with no adjunctive therapy. However, injecting purified ME cells into nude mice induced a mixed mammary tumor with metastasis. These results are contrary to each other. Over the past two decades, the role of ME cells in mammary carcinogenesis remains controversial. Much evidence has shown that normal ME cells act as “natural tumor suppressors” through paracrine regulation and microenvironmental alteration of the tumor [36, 37]. Nevertheless, Lo et al. found that tumor-associated ME cells may actually promote tumor invasion through the TFG- β signaling pathway [38]. It is also hypothesized that the tumor suppressive effect of myoepithelial cells is dependent on its differential stages [18, 39]. In the present study, ME cells acted as tumor promoters. This phenomenon may be related to the stem cell property/undifferentiated stage of the myoepithelium, increased HER2 expression, and potential spontaneous EMT process. However, further studies need to be conducted to elucidate the role of ME cells in mammary carcinogenesis and hopefully to determine their prognostic value.

3.2 CMT-1 experienced spontaneous EMT

EMT has a dual nature. In normal development, EMT and its reverse process MET are critical for embryogenesis. However, in cancer, it is associated with tumor stemness, metastasis, and resistance to therapy [40]. Researchers found that multiple subpopulations with different EMT stages in primary

mammary tumors present differences in cellular plasticity, invasiveness, and metastatic potential. Cellular plasticity varied and invasive capacity increased with the degree of EMT while metastasis was enhanced in the hybrid EMT state [21].

E-cadherin is the prototypical cell marker of EMT, expressed in epithelial cells. Vimentin is another classical cell marker of EMT, expressed in mesenchymal cells. During EMT, the expression of E-cadherin decreases while that of vimentin increases [41]. Our results showed that, compared with CMT-1 (passage 5), a significant increase in vimentin and decrease in E-cadherin in CMT-1 (passage 50) was noted in both qPCR and WB. Moreover, metastatic lesions were found in the diaphragm in our transplantation model. This suggested that CMT-1 had undergone a spontaneous EMT process where CMT-1 (passage 5) lost its epithelium-like phenotype and CMT-1 (passage 50) adopted mesenchymal cell characteristics. The spontaneous EMT property of CMT-1 cells may be utilized to further elucidate canine mammary carcinogenesis.

3.3 CMT1 cells with stem cell property formed the first canine mammosphere

Instead of placing cells onto a flat surface, 3D culture provides a scaffold for cells to attach to during culture. When applying stem cells on 3D culture, cells will divide into several cell subpopulations and undergo self-organization to form a so called “organoid” [42]. Thanks to its characteristics, 3D culture allows more natural cell growth and cell-cell interactions, resembling the *in vivo* environment, to better understand the actual developmental biology or disease progression. Apart from these, organoids have other applications such as drug discovery and screening, personalized medicine, and regenerative medicine [43, 44]. In addition, this new culture method improves research efficiency and reduces animal use.

In human medicine, a variety of organoids from different tissues have been established, including optic cup and retina, cerebellum, lung, small intestines, liver, pancreas. [42, 45, 46]. However, only a few canine organoids have been reported. For instance, a canine prostate cancer organoid and canine bladder cancer organoid were established using urine samples [47, 48]; a canine keratinocyte organoid and epidermal organoid were developed utilizing hair follicles [49, 50]; canine intestinal organoids were derived from the intestinal stem cells of both healthy and gastrointestinal-diseased dogs [51]. Despite the high morbidity of CMT, the development of a CMT organoid for better understanding the disease process has not been reported. On the other hand, the use of human mammary gland organoids has advanced owing to a recently published protocol [52]. Although bovine mammary gland organoids have been produced using primary bovine mammary epithelial cells from fresh milk [53], considering species differences and disease predisposition, this may not be an optimal model for CMT study.

In the present study, we demonstrated the stemness of the CMT-1 cell line by successfully producing a canine mammosphere, which displayed several cell morphologies including grape-like cell clusters, spheroid solid cell balls, and single cystic/luminal structures. With this mammary tumor organoid, we

may elaborate the tumorigenesis of CMTs and screen for new drugs to combat tumor resistance in the future. To the best of our knowledge, this is the first documentation of a canine mammosphere.

4 Conclusion

In this study, we successfully established a novel HER2-enriched CMT myoepithelial cell line and a canine mammosphere with *in vitro* and *in vivo* study. The development of the CMT-1 cell line and organoid is valuable for the study of tumorigenesis and resistance of CMT. With stem cell and spontaneous EMT properties, the CMT-1 cell line may be a good candidate for decoding the EMT mechanism. As the techniques mature, CMT-1 organoids could serve as platforms for anti-HER2 drug screening.

5 Materials And Methods

5.1 Establishment and purification of primary cultures

Mammary tumor tissues were cut into small pieces ($1 \sim 3 \text{ mm}^3$) and washed three times with phosphate-buffered saline (PBS; Biological Industries, Kibbutz Beit-Haemek, Israel) containing 1% penicillin-streptomycin solution (Beyotime, Shanghai, China). Tumor samples were digested with TrypLE Express (Gibco, Grand Island, NY, USA) for at least 6 h. The digested tissue suspension was collected and placed in a 15 mL centrifuge tube for centrifugation at 1000 rpm for 10 min. The cells at the bottom were resuspended in Dulbecco's modified Eagle medium (DMEM; Biological Industries) with 20% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin solution (Beyotime). The cells were placed on 12-well plates and cultivated at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO₂). Cells were observed daily by microscopy. When the cell cultures reached 90% confluence, the cells were incubated with TrypLE Express. The digested cells were transferred into new 25 cm² flasks at a density of $\sim 1 \times 10^5$ cells/mL. Cohorts of cells were frozen at every five passages. CMT-1 was considered as an established cell line after passage 50.

5.2 Growth studies and doubling time

CMT-1 cells (passage 50) were cultured on 12 well plates, with 5×10^3 cells per well, and maintained in DMEM supplemented with 10% FBS for 10 d. At 24-h intervals, three replicative wells were digested and counted with Countess 3 (Invitrogen, Carlsbad, CA, USA). Cell growth doubling time was calculated from the exponential growth phase.

5.3 Wound healing assays

In cell migration assays, CMT-1 cells (passage 5 and passage 50) were seeded on 12-well plates. The cell motility was assessed by measuring the movement of cells into a scarp. The speed of scarp closure was monitored after 12, 24, 36, and 48 h by measuring the ratio of the distance of the scarp from 0 h. Each experiment was performed in triplicate.

5.4 Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from CMT-1 cells at passage 5 and passage 50 using a Simply P Total RNA Extraction kit (Bioer Technology, Hangzhou, China) according to the manufacturer's instructions. cDNA samples (1000 ng) were prepared from total RNA using a HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qRT-PCR was carried out on a LightCycler 480 (Roche, Basel, Switzerland) with ChamQ SYBR qPCR Master Mix (Vazyme) using the following program: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Samples were analyzed in triplicate and three independent experiments were performed. The mRNA expression levels of target genes relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were calculated with the $2^{-\Delta\Delta C_t}$ method and plotted as fold changes compared between cells from passage 5 and passage 50. Primers used for qRT-PCR were designed based on published sequences and are listed in Supplementary Table S1.

5.5 Western blotting (WB)

CMT-1 (passage 5) and CMT-1 (passage 50) cells were seeded on 6-well plates, respectively. After 100% confluence, the cells were lysed using Cell Lysis Buffer for Western blot and IP (Beyotime). A total of 30 µg of each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane that was blocked for 15 min at room temperature (RT) with QuickBlock Blocking Buffer for Western Blot (Beyotime) and then incubated overnight at 4°C with primary antibodies, followed by a 1 h incubation with a secondary antibody. Protein bands were visualized by Odyssey Sa (Li-cor, Lincoln, NE, USA). The primary and secondary antibodies used in this study are listed in Supplementary Table S2.

5.6 Indirect immunofluorescence analysis (IFA)

CMT-1 cells (upon passage 50) were seeded on a 12-well plate with glass slides. The cells were washed three times with PBS and then fixed in cold 4% paraformaldehyde at RT for 10 min. For immunofluorescence staining, cells were blocked for 10 min at RT (QuickBlock Blocking Buffer for Immunol Staining; Beyotime). Then the cells were incubated overnight at 4°C with the primary antibody and for 1 h at RT with the secondary antibody. The nuclei were stained with DAPI (Beyotime) for 5 min. The fluorescence signal was observed with a confocal laser scanning microscope (SP8 TCS; Leica, Wetzlar, Germany). Primary and secondary antibodies used in this study are listed in Supplementary Table S2.

5.7 Histopathology and immunohistochemistry (IHC)

For histopathological assessment, representative tumors and other tissues with lesions were fixed in 10% buffered formalin, processed by routine methods, embedded in paraffin wax, sectioned at 3 µm and stained with hematoxylin and eosin (HE). Histopathological findings were recorded and used to classify the tumors according to Goldschmidt et al. [24]. For IHC assessment, the tissue samples including CMT-1 induced tumors, lungs, and diaphragm were prepared in 3 µm samples. Tissues were deparaffinized for 20 min and underwent heat-induced antigen retrieval with high pressure-heated methods in sodium citrate buffer (pH 6.0) for 3 min, then cooled to RT. The slides were covered with 3% hydrogen peroxide for 10 min to terminate the peroxidase activity. Specimens were then incubated with primary antibodies

(listed in Supplementary Table S2) at 37°C for 60 min and with secondary antibody (BOND Polymer Refine Detection; Leica Biosystem, Buffalo Grove, IL, USA) at 37°C for 1 h in order to detect target proteins. Each specimen was washed three times with PBS. Signal was visualized using 3,3'-diaminobenzidine (DAB). The samples were counterstained with hematoxylin, dehydrated, cleared, and mounted. Interpretation of HER2 staining was performed according to the Herceptest™ interpretation manual.

5.8 Transplantation into mice

For tumorigenicity assays, a randomized analytic experiment was performed. Fifteen 8-week-old female BALB/c nude mice (Vital River Laboratory Animal Technology, Beijing, China) with a mean body weight of 20.06 g were included. The study was carried out in compliance with the ARRIVE guidelines and was approved by the Institutional Animal Care and Use Committee of South China Agricultural University (Permit Number: 2021B102). All mice were randomly separated into groups A, B, and C. Each group contained five mice. A 0.2 mL DMEM suspension of CMT-1 (passage 50) containing 1×10^7 cells was subcutaneously inoculated into the left inguinal mammary fat pads of mice in group A and B. Mice in group C were injected with 0.2 mL saline per mouse at the same location, as the negative control. The growth of tumors was monitored and measured weekly and the tumor size was calculated using an equation $V = 1/2 \times L \times W^2$ (V = volume, L = length, W = width). Mice were sacrificed by cervical dislocation at 61 d after inoculation based on American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2020 Edition. The tumor masses and organs were collected. Neoplasms and organs were collected in 4% paraformaldehyde for histopathological detection and IHC.

5.9 Three-dimension organoid culture

The digested and single cells of CMT-1 cells were resuspended in cold Cultrex Reduced Growth Factor Basement Membrane Extract (Type 2, Select; Bio-Techne, Minneapolis, MN, USA) and seeded in a prewarmed 24-well plate (at 25 μ L drops per well). The drops were solidified in a 37°C and 5% CO₂ incubator for 30 min, and then 0.5 mL of organoid culture medium (Accuroid medium; Accurate International, Guangzhou, China) was added to each well and refreshed every 3 d.

5.10 Statistical analysis

Relative expression levels are presented as mean $\pm$ standard deviation. The statistical significance of differences was evaluated with the unpaired Student's t-test using Prism v6.0 software (GraphPad, La Jolla, CA, USA) (mean $\pm$ standard deviation (SD), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Abbreviations

CMTs: Canine mammary tumors, ER: estrogen receptors. PR: progesterone receptors, HER2: human epidermal growth receptor-2, EMT: epithelial to mesenchymal transition, IHC: immunohistochemistry, HBC: human breast cancer, EGFR: epidermal growth factor receptor, ME: myoepithelial, α -SMA: α -smooth

muscle actin, CSCs: cancer stem cells, WB: western blotting, IFA: Immunofluorescence assay, GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Declarations

Ethics approval and consent to participate

The Institutional Animal Care and Use Committee of South China Agricultural University (Guangzhou, Guangdong Province) approved the experimental procedures in the present study (Permit number: 2021B102).

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

SL supervised all experiments. AC designed the experiments and was the major contributor in writing the manuscript. JZ conducted the animal experiment. SY performed practical work and analyzed the dataset. JL, ZC, and YZ assisted with the experiments. All authors read and approved the final manuscript.

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Figures

Figure 1

Histopathology of the CMT with H&E and IHC staining.

(A) Uncapsulated mass composed of proliferative mammary epithelial and myoepithelial cells. Scale bar = 200 μ m. (B) Nuclei were oval to elongated containing finely stippled scant chromatin and indistinct nucleoli. Scale bar = 50 μ m. IHC staining images showed that CMT was ER negative (C), PR negative (D), HER2 positive (E), α -SMA positive (F), and E-cadherin positive (G). Scale bar = 200 μ m.

Figure 2

Growth studies and doubling time of the CMT-1 cell line. (A) CMT-1 cells demonstrated moderate anisocytosis and anisokaryosis with the majority of cells having multiple nuclei. (B) CMT-1 cells (passage 9) showed slight anisocytosis and anisokaryosis with the majority of cells having a single nucleus while CMT-1 (passage 50) became more uniformly round to oval in morphology. (C) The population doubling time of CMT-1 was calculated to be 26.67 h.

Figure 3

Western blotting results of different cell lines. The results of western blotting of MDCK cells, CMT-1 cells (passage 5), CHMp cell line, and CMT-1 cells (passage 50) (ER, PR, HER2, and β -actin). Full-length blots/gels are presented in Supplementary Figure 1A-D.

Figure 4

Molecular subtype of the CMT-1 cell line. The results of immunofluorescence assay of the CMT-1 cell line (ER, PR, HER2, E-cadherin, Vimentin, and α -SMA).

Figure 5

Migration assay. (A) Cell migration ability was tested at 24 and 48 h. The black vertical lines in the images define the open wound areas. (B) The average migration distance of various preparations are shown. All assays were independently repeated at least three times. Ns indicates not significant.

Figure 6

Expression of E-cad, and vimentin of CMT1 cells. (A) WB for E-cadherin and vimentin. MDCK cells showed weak reaction to E-cadherin while CMT-1 cells (passage 5) strongly expressed E-cadherin. CMT-1 cells (passage 50) lost E-cad expression but gained greater vimentin expression. (B) Relative fold changes in E-cadherin and vimentin expression using qPCR between CMT1-P5 and CMT1-P50. All experiments were independently repeated at least three times. The data are shown as the mean $\pm$ SD, $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Full-length blots/gels are presented in Supplementary Figure 2A-C.

Figure 7

Representative bright-field images of different morphologies of CMT-1 mammary organoids. Black arrow: grape-like, Blue arrow: solid, Red arrow: cystic. Scale bar = 50 μ m (indicated in the bottom right corner of the image).

Figure 8

Nude mouse tumorigenicity assay. (A) The primary tumor appeared as a small solid mass at the injection site. Diffuse to coalescing whitish lesions (blue arrow) were noted on the diaphragm. (B) Tumor volume of the nude mice. The data are shown as the mean $\pm$ SD, n = 10. (C) Epithelial cells formed nests and irregular tubules on a spindoloid stromal proliferation in a primary xenograft tumor. (D) Diaphragmatic muscle degeneration and atrophy adjacent to the neoplastic cell infiltrates.

Figure 9

IHC of the xenograft tumor and the metastatic diaphragm. Both the xenograft tumor and metastatic diaphragm were negative for ER and PR, but positive for HER2, α -SMA, and E-cadherin. The metastatic diaphragm had stronger staining for HER2.

Supplementary Files

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