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# Establishment and Characterization of Canine Mammary Gland Carcinoma Cell Lines with Vasculogenic Mimicry Ability *in vitro* and *in vivo*

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## ABSTRACT

Mammary neoplasms affect a population of uncastrated and elderly female dogs and most of these neoplasms are malignant. In order to study this disease cell culture presents itself as a promising preclinical model, creating the opportunity to deposit cell lines at a cell bank, allowing a great repetition of the assays and making the validation of the results more reliable. Including, *in vitro* experiments for vasculogenic mimicry (VM) evaluation. VM is related to cancer cells capable of generate vascular-like structures without endothelial cells, mimicking the vasculogenic process. The aim of this study was to establish and characterize ten cell lines from canine mammary gland tumour according to immunophenotype and tumorigenicity, and with its ability to form vasculogenic mimicry-like structures in vitro. Fifteen samples from canine mammary gland carcinoma were collected and cultured in vitro and ten cell lines were established and characterized. Cells were evaluated for morphology, phenotype, vascular mimicry and tumorigenicity. All cell lines presented spindle shape morphology and expressed concomitant pan-cytokeratin and CK8/18. Four cell lines had vasculogenic mimicry ability and two cell showed in vivo tumorigenic potential. Cell characterization of those lines will help to create a database for more knowledge of mammary gland carcinomas in dogs, including studies of tumor behavior and new therapeutic targets.

**Keywords: female dog; cell culture; mammary cancer; veterinary; oncology**

## BACKGROUND

Intact and old female dogs are frequently affected by mammary gland tumours and more than 50% of the cases are malignant [1, 2]. In women, breast cancer is the main cause of mortality and the most common cancer type diagnosed [3]. The occurrence of neoplasms in dogs are spontaneous and similar to breast cancer in women, as histological appearance, molecular targets and biological behaviour. For these reasons the canine mammary gland tumour is an experimental model for human breast cancer [4].

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Mammary carcinomas in female dogs need more understanding, once chemotherapy treatment is not as effective as in humans and don't increase the patients survival time or influence local disease control [5, 6]. In dogs, the golden standard treatment it is radical surgery and chemotherapy is based on the human breast cancer literature [7, 8]. Thus, even performing chemotherapy in female dogs affected by mammary gland tumours, there is no standardized protocol [6]. Besides that, we lack information regarding markers that can predict antitumor response, as occurs in human breast cancer treatment [9, 10]. In this scenario, cancer cell lines represent a great start in the evaluation of antitumor response.

Cell lines are an alternative and also an experimental model *in vitro* to human breast cancer and canine mammary gland tumours, allowing the investigation of carcinogenesis process as proliferation, apoptosis and migration [11]. Cell culture is considered an excellent preclinical model, essential for the identification and evaluation of drugs action mechanism, identification of genes involved in carcinogenesis, such as oncogenes and tumour suppressors, defining cell signalling pathways and how they contribute to tumour pathogenesis, discovering of new drugs and for the development process of antitumor drugs [12].

In canine and human patients affected by highly aggressive mammary neoplasms, neoplastic cells can be able to form vascular-like structures or channels, able to conduct plasma, red cells and neoplastic cells during epithelial mesenchymal transition [13]. Thus, vasculogenic mimicry (VM) have been widely studied as a mechanism of tumour nutrition and metastasis [13, 14, 15, 16]. In canine mammary gland tumours, the phenomenon has been studied in inflammatory mammary carcinomas and other tumour subtypes. However, to the best of our knowledge, there is no previous study establishing or evaluating canine mammary cancer cells ability to form vasculogenic-like structures as an in vitro model of canine mammary carcinomas. Thus, this study aimed to establish and characterize nine cell lines from canine mammary gland tumour according to immunophenotype, tumorigenicity and ability to form vascular-like structures in vitro and in vivo.

## MATERIALS AND METHODS

### Reagents

All reagents used had high purity and were purchased from companies GE Healthcare (Uppsala, Seeden), Sigma-Aldrich (São Paulo, Brazil), Merck SA (São Paulo, Brazil), otherwise, they were cited. In addition, cell culture medium was used *Mammary Epithelial Cell Growth Medium* (MEGM™, Lonza Inc., Allendale, NJ, USA), medium *Dulbecco's Modified Eagle Medium:F12* (DMEM/F12, Lonza Inc., Allendale, NJ, USA), fetal bovine serum (FBS, LGC Biotecnologia, Cotia, SP, Brazil), *Dulbecco's Phosphate Buffered Saline* (DPBS) (Sigma Aldrich, St. Louis, MO, USA), antibiotic/antimycotic solution (TermoFischer Scientific, Waltham, MA, USA) and trypsin (0.25%, GBICO TermoFischer Scientific, Waltham, MA, USA).

### Cell samples and isolation

Fifteen samples of mammary gland tumours were collected at the veterinary teaching hospital (between December 2016 and March 2017) for histopathological examination and the samples were expanded in cell culture. Cell lines were obtained using enzymatic dissociation as previously described in the literature [17]. Briefly, tumour fragments with approximately 1 $cm^2$ were collected and dissociated with type IV collagenase (Sigma-Aldrich, St. Louis, MO, United States) for four hours at 37 °C, in a humidified atmosphere containing 5% $CO_2$. After this process the

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material was separated using a 75 μm mesh filter, centrifuged and washed with Dulbecco's Phosphate-Buffered Saline DPBS (Sigma-Aldrich, St. Louis, MO, United States) to remove excess collagenase. After cell isolation process, they were counted in a Neubauer chamber and the cell viability was evaluated by the Trypan blue technique. Plating was performed at a concentration of $10^4$ cells / mL in 25 mL culture bottles with filter.

Selected samples, histological subtypes, and cell obtention technique are described in Supplementary Table 1. Identification of histological subtypes and tumour grade were based on the international classification of mammary gland tumours [18].

**Molecular phenotype of primary tumours and respective metastasis**

Immunohistochemistry (IHC) was performed with the tumour's samples from paraffin block, wherein 4 μm-thick sections were placed onto positively charged slides (StarFrost, Braunschweig, Germany) and then, deparaffinized. To antigen recovery, citrate buffer (pH 6.0) was used in a pressure cooker (Pascal, Dako, Agilent Technologies, Santa Clara, CA, USA) and endogenous peroxidase was blocked with 8% hydrogen peroxide (Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil) in methanol (Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil) for 20 min. Nonspecific binding was blocked with 8% skim milk for 60 min, both at room temperature. The antibodies for HER2, ERα, PR, Ki-67, CK5/6 and EGFR and respective clones were diluted and samples remained incubated according to Nguyen et al. [19] (Supplementary Table 1). Antibody detection was achieved using a polymer (EnVision, Agilent Technologies, Santa Clara, CA, USA). 3,3'-Diaminobenzidine (DAB) (EnVision, FLEX, High pH, Dako, Agilent Technologies, Santa Clara, CA, USA) was used as a chromogen and tissue counterstaining was performed with Harris Haematoxylin. Positive [19] and negative [20] controls were performed according to the previous literature.

ERα and PR were considered positive when ≥ 10% of the nuclei were stained, CK5/6 and EGFR receptors when the cytoplasm staining was ≥ 10% and Ki-67 when more than 33.3% of the cells had stained nuclei. For the evaluation, a group more than 500 cells were counted [19]. HER-2 staining was scored for labelling distribution (0: unlabelled or <10% labelled tumour cells; 1+:> 10% of labelled tumour cells with incomplete membrane labelling; 2+: moderate to > 10% strong tumour cells; 3+: complete and strong membrane labelling of > 10% tumour cells). In this classification, 0 and 1+ are considered negative and, 2+ and 3+ positive.

**Elimination of contaminating fibroblasts**

To eliminate fibroblasts from primary cultures, selective cell trypsinization with cold trypsin (4°C) was performed according to the previous literature [21] at passage 5 (P5). Briefly, cells were washed with DPBS at 4 °C to avoid direct thermal shock by the cold trypsin. Subsequently, 2 mL of cold trypsin (4 °C) were used at room temperature (27 °C) for 2 min. Then, supernatant was inactivated with complete medium containing 10% FBS in a 1: 1 ratio and trypsin was collected and discharged. Then, the flasks were washed with DPBS buffer to remove residual trypsin. Subsequently, cells were washed with DPBS solution at room temperature twice, and then washed with DPBS buffer at 37 °C once. After this procedure, 800μl of trypsin at 37 °C were added to the bottle and the cells were kept in a humid atmosphere at 37 °C in a humidified atmosphere containing 5% CO2 for 5 min. After this period, the remaining cells were detached with manual mechanical impact and the trypsin was inactivated with complete medium

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(containing 10% FBS) in a 1:1 ratio. Then, cells were centrifuged for 5 min at 1200 rpm and resuspended in 5 ml of MEGM™ medium containing 10% FBS and 1% antibiotic and antifungal.

**Cell Expansion**

Cell culture was stablished from tumour fragments in MEGM medium containing 1% antibiotic/antimycotic solution and 10% FBS kept in a humid atmosphere containing 5% CO2 at 37º C. When the cells reached 80% confluence, the medium was discarded and the bottles were washed with sterile DPBS pH 7.2 to eliminate residual FBS. For detachment of the cells from the bottle, trypsin 0.25% was added at 37 °C, followed by a 5 min incubation period in a 5% CO2 humid atmosphere and 37 ºC. Cells were cultured until 10$^{th}$ passage and used for cell phenotyping, karyotype, morphology and western blot.

**Cell Karyotype**

Karyotype analysis was performed according to Moorhead et al. [22]. Initially the cells at P10 were cultured in DMEN/F12 medium, supplemented with FBS (10%) and phytohemagglutinin for 72 hours. After this period, the cells were evaluated under the inverted microscope and the mitotic spindle was interrupted by the addition of colchicine (16 μg / ml). Subsequent washes and centrifugations were performed at 4 °C to then fix the slide material and stain by the Wright-Giemsa staining method for karyotype assembly.

**Cell morphology and doubling time**

The same procedures performed for immunofluorescence and identification of cell phenotype were conducted to evaluate cell morphology until permeabilization. Subsequently, the permeabilizing solution was removed and the cells washed three times with DPBS. After that, cells were stained with hematoxylin and eosin (HE).

Cells were also evaluated for cell doubling time. The cell medium was discarded and the bottles were washed with sterile DPBS pH 7.2 to eliminate residual FBS. For detachment of the cells from the bottle, trypsin was added at 37 °C, followed by a 5 min incubation period in a 5% CO2 humid atmosphere and after this period, trypsin was inactivated with cell culture medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution. Cells were centrifuged (450 g, 5 min) and after discarding the supernatant, resuspended in 1 mL DMEM/F12 medium with 10% FBS and 1% antibiotic/antimycotic solution. Cells were diluted in Trypan blue (Trypan blue solution, cod. T8154, Sigma-Aldrich, St. Louis, MO, USA) in a 1: 1 ratio and counted in a Neubauer chamber. For doubling time, the protocol described by Caceres et al. [23] was followed. Briefly, 1x10$^5$ were plated in 25cm$^2$ flasks and maintained with DMEN/F12 medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution in triplicate. Every 24 hours cells were trypsinized and counted. This procedure was performed for 5 consecutive days to evaluate the exponential growth curve. The final amount of cells for each culture was obtained by averaging three counts.

**Cell Phenotype**

The phenotype of each culture was evaluated at P10 to characterize the cell clone that grew in each culture. For this, 12 sterile well plates with sterile circular coverslips were used and placed in the centre of each well. After plate preparation, 500 μl of complete culture medium were added to each well and subsequently 10$^3$ cells were pipetted into

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the middle of the well for 72 hours. After this period cell density in each coverslip was verified. When the coverslips presented confluence above 50% they were removed for immunofluorescence (IF).

For the IF technique the medium was removed, the coverslips washed with DPBS three times and then fixed with cold methanol absolute (4 °C) for 30 min in a refrigerator (8 °C). After methanol was removed, the cells were washed three times with PBS and immersed in 0.1% Triton-X solution for 10 min at room temperature for cell permeabilization. Then, cells were blocked with commercial solution (Protein block, Dako, CA, USA) for 30 min at room temperature and primary antibodies were added to each well. We investigated the pan-cytokeratin, cytokeratin 8/18 and vimentin expression. The information regarding the antibodies can be found in Supplementary Table 3. Cells were incubated with goat anti-mouse IgG secondary antibody (Alexa Fluor 647, Life Technologies, Corporation, Carlsbad, CA, EUA) and counterstained with DAPI (Sigma Aldrich, St. Louis, MO, EUA) at 1:10.000 dilution. As a negative reaction control, the primary antibodies tested during the procedure were omitted and replaced with TRIS buffer solution.

**Tubular formation *in vitro***

Tubular formation *in* vitro it is an assay to identify a vasculogenic mimicry ability in culture conditions. For tubular formation assay the cells were cultured in three-dimensional (3D) conditions. After each cell culture achieved more than 80% of confluency, they were trypsinized and $50x10^3$ viable cell were cultured with DMEM in a 24-well plate with 250 µL of Matrigel (Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, *LDEV-Free, Corning, New York, NY, USA). The Matrigel was added in each well and air-dried for 30 min, at room temperature. The cells were incubated in a humidified atmosphere with 5% of $CO_2$ at 37°C. Then, the cells were evaluated in an inverted microscopy every hour to determine tubular formation. The experiment was performed in triplicate for each cell culture.

**Tumour growth in immunodeficient mice**

To evaluate cell culture tumorigenicity, a total of 12 nude mice (BALB/c nude, C.Cg-Foxn1nu line) were acquired from the Institute of Biomedical Sciences, University of São Paulo – USP and housed in individually ventilated caging. All procedures regarding feeding, humidity, temperature and light control were performed according to the literature [24]. For in vivo tumorigenicity evaluation, $1x10^6$ cell were inoculated on subcutaneous mammary gland region and the subjects were accompanied once a week, for at least 60 days. After tumour growth, the visits occurred daily to measure tumour volume. After achieving 3 $cm^2$, the subjects were submitted to humanely euthanasia and the material from the tumour was collected to confirm malignancy with histological evaluation. Then, pan-cytokeratin and vimentin immunohistochemistry was performed to confirm tumour phenotype. The immunohistochemistry analysis was performed as described above, using the antibodies mouse monoclonal anti-vimentin (Clone V3, Santa Cruz Biotechnology, Dallas, TX, USA) and mouse monoclonal anti-cytokeratin (Clone AE1/AE3, Santa Cruz Biotechnology, Dallas, TX, USA) at 1:300 dilution, overnight. The secondary antibodies, chromogen, counterstaining and negative controls were performed as described above. The epithelial component of a normal skin was used as positive control for pan-cytokeratin and the endodermis was used as positive control for vimentin.

**RESULTS**

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**Molecular phenotype of primary tumours and respective metastasis**

Of all the fifteen tumour samples, only the ones that were able to grow on cell culture were evaluated by IHC. Six were classified as HER-2 overexpressing (CM1, CM9, CM11, CM60, MM3 and MM4) and 3 as triple negative (CM5, CM61 and MM1).

**Samples acquisition and cell isolation**

Ten out 20 samples grew *in vitro* and the different tumour classification, graduation, acquisition technique, time exposed to collagenase and passage are described in table 1. The samples CM2, CM3, CM6, CM7, CM8, CM10 and CM12-15 did not shown *in vitro* expansion. The cell cultures CM2 and CM3 were cultured using enzymatic dissociation overnight and had no cellular growth after 72 hours in culture conductions. The culture of CM6 and CM7 cells were made using explants and for both cell lines, fungal contamination discontinued the cellular growth. Thus, cells submitted enzymatic dissociation with type IV collagenase showed the best *in vitro* expansion.

**Contaminating Fibroblast Elimination**

Cell cultures prior P5 showed mixed morphology (spindle cells, polygonal cells, cells growing in groups, and rounded cells) (Figure 1A). After selective trypsinization, cells from trypsinization at 4 °C showed were grow and showed slow growth with spindle morphology (Figure 1B) and cells did not show confluence above 50% after 30 days of cultivation and were discharged. Cells from trypsinization at 37 °C showed homogeneous morphology with some cultures presenting uniform spindle morphology (CM1, CM4 and MM1) or polygonal morphology (CM5, CM9 and CM11) (Figure 1C). Cells were grown to passage 10 (P10), showing approximately 90% confluence 48 hours after passage.

**Cell Karyotype**

After karyotype preparation and staining, 70 different images were captured for each cell culture and at least 20 metaphases of each culture were analysed, according to the previously described by Gouveia et al. [25]. Of the 70 images captured, the best ones for chromosome counting were selected (Supplementary Figure 2). It was possible to identify aneuploidies in different metaphases of different cell cultures. In cell cultures CM4 and CM11, hypoploidy of metaphase was identified, as well as hyperploid of cultures CM1, CM60 and MM3. The representation of chromosomal alterations in each culture can be seen in Supplementary Figure 2.

**Cell morphology, phenotype and doubling time**

In the morphological evaluation (Figure 3I and 3J), the primary and metastatic adenosquamous carcinoma tumour cells showed growth in monolayers, in fusiform pattern, basophilic nucleus and eosinophilic cytoplasm, with presence of mitosis. Grade III solid carcinoma cells showed multinucleated cells and colony formation (Figure 3K and 3L). All samples evaluated showed strong pan-cytokeratin (Figure 4A) and CK8/18 (Figure 4B) staining and were negative for P63 (Figure 4C). All cell cultures also showed vimentin positive cells (Figure 4D). The concomitant pan-cytokeratin and CK8/18 expression for all cell lines confirmed their epithelial phenotype. The primary culture cells, CM1, CM4, CM5, CM9, CM60 and CM61 had doubling time at 6.31, 5.1, 7.5, 8,95, 11.79 and 13.06, respectively. The MM1, MM3 and MM4 metastasis cultures presented their doubling time at 25.41, 34.17 and 10.28, respectively.

 doi:10.20944/preprints201912.0162.v1

**Tubular formation *in vitro***

The in vitro tubular formation was identified in 4 out of 9 cells. The cell lines CM1, CM9, CM60 and MM4 presented tubular formation at four hours until six hours and tubules were disrupted from 6 hours (Figure 5).

**Tumour growth in immunodeficient mice**

One primary cell culture (CM60) and its respective metastasis (MM4) showed in vivo tumorigenicity (2 out of 10 cell cultures). The macroscopic growth was evident after 50 days after cell application and histology revealed a tumour with highly vasculogenic mimicry formation. Both cell lines presented neoplastic cells with evident nucleoli forming tubular structures like capillaries. These capillaries-like structures were positive for both pan-cytokeratin and vimentin, indicating. Interestingly, the metastatic cell line (MM4), also showed an intravascular growth. It was possible to observe several blood vessels in tumour periphery with intravascular growth of cancer cells positive for pan-cytokeratin.

## DISCUSSION

Canine mammary gland tumours represent one of the most important tumours in intact female dogs and can be considered a model for studying human disease. In human medicine, several cell lines have been used as a preclinical model for understanding breast cancer (BC) development and progression or studying the antitumor effect of new drugs [26, 27, 28, 29]. Comparing with human BC, there is much less canine mammary gland tumour cell line developed and studied [24, 30, 31, 32]. To establish the primary cell cultures, we tried different methodologies aiming to identify the most effective in isolating the neoplastic epithelial mammary cells. The cultures obtained through explant samples presented several problems in their establishment, mainly related to contamination during cultivation. Thus, we tried protocols based on the use of enzymatic dissociation. In this step we tried collagenase type I and II in different incubation time (3, 4, 12, 24 and 48 hours) with no success (data not shown). On the other hand, using 0.05% type IV collagenase we had better results. For type IV collagenase 0.05% with 24- and 48-hours incubation, we identified cell damage and death, with no cell growth (data not shown). For samples incubated using 0.05% type IV collagenase within 4 hours there was no cell damage and satisfactory cultures expansion. Therefore, we standardized the time to up to 4 hours for enzymatic dissociation.

After the different cell cultures were established, they presented a heterogeneous morphology, so selective trypsinization of cells was performed to eliminate fibroblasts and stimulate cell clone formation. It was possible to observe the formation of cell clones and then the cells became homogeneous and easily multiplied [33]. The cells were used from passage 10 (P10) after initial expansion, since from higher passages they tend to have epithelial morphology, ability to form agglomerations and multiply easily, with the same pattern of cellular phenotype. In addition, from several passages contamination with fibroblasts that grow concomitantly with tumour cells used to be eliminated [34, 35]. Cell lines presented monolayers growth, similar size and morphology. In addition, they showed spindle-shape, high nucleus/cytoplasm ratio and a tight cell-cell adhesion. The high ratio between nucleus and cytoplasm size can usually be associated with malignant tumour behaviour [36]. In hematoxylin and eosin staining, it was possible to observe basophilic nucleus and eosinophilic cytoplasm, with presence of mitosis and some cells appeared multinucleated and with colony formation.

Concomitant expression of pan-cytokeratin and CK8/18 confirmed the epithelial origin of our canine mammary tumour cells, reinforcing that our protocol was effective in isolating these cells. Besides that, our cell lines

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also expressed vimentin, same results found in MCF7 and HeLa cells cultured *in vitro* [37, 38]. Since in culture conditions cells need to change their cytoskeleton for the flask attachment, it is common to observe vimentin expression either in epithelial cells [39]. This expression was previous explained by post translational modification during cell culture conditions [23]. Doubling time of each culture was analysed and the shortest was from CM4 (5.1) and the longest from cell MM3 (34.17) at passage 10. This is important, considering that the cell doubling time should vary with passages. The difference in cell doubling time is expected as these are different tumours. These results are similar to those found by Cordeiro et al. [24] in which the doubling times of two different cells lines cultured *in vitro* were 26 and 42 hours and, in this case, one cell line was more malignant than other.

An interesting finding in this study was the tubular formation *in vitro* in four different cell lines, that can be related to vasculogenic mimicry (VM). Tumours with VM capacity can help to promote metastasis of tumour cells through blood vessels. Also, tumour cell lines are highly malignant and are capable of penetrate endothelium, developing tumour invasion and metastasis [15]. Among these cells, two also showed vasculogenic mimicry ability in vivo. The ability of VM formation is associated with aggressive melanoma cells and not with non-aggressive cells [16], and high tumour grade, invasion, metastasis and poor clinical prognosis in hepatocellular carcinomas [14]. In inflammatory mammary tumours, both human and canine, VM is commonly described, possibly related to tumour aggressiveness and metastasis capacity [40, 41]. The VM results corroborate with the results from tumorigenicity assay where two cellular types, that were capable of VM formation, produced tumours in vivo. When analysed in HE, the tumours in vivo had highly vasculogenic mimicry formation, showing the aggressivity of this neoplasia, in vivo and *in vitro*. The ability to form vasculogenic mimicry in vitro and in vivo, reinforce the use of these cells as a preclinical model for canine mammary gland tumours.

## CONCLUSION

In summary, this study had established and characterized 10 cell lines and xenografts from canine mammary gland carcinomas. The cells cultured *in vitro* demonstrated morphological and phenotype similarities, but had tumorigenicity differences. Furthermore, four cell lines were capable of vasculogenic mimicry ability and two cell lines showed in vivo tumorigenicity, both related to malignancy and aggressiveness. Thus, described cell lines will be able to be used in the future for clinical investigations, therapeutic targets, and also for study genes targets and pathways. The results showed similarities with BC in human demonstrating that the results obtained with these cell lines may be extrapolated to humans.

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