



Assessment of the tumorigenesis and drug susceptibility of three new canine mammary tumor cell lines

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ARTICLE INFO

Article history:

Accepted 19 August 2009

Keywords:

Canine
Mammary tumor
Anti-tumor drugs
Tumorigenesis

ABSTRACT

Three canine mammary tumor (CMT) cell lines, namely DE-E, DE-F and DE-SF, have been established from a surgically excised specimen of a malignant mammary tumor. These CMT cell lines have been cultured for over 200 passages. The cell doubling time was estimated to be approximately 30 h for all three cell lines. DE-E, DE-F and DE-SF were epithelial, fibroblast and spindle fibroblast in morphology, respectively. Under electron microscope, DE-F and DE-SF cells displayed a higher nucleus/cytoplasm ratio as compared with DE-E. Variation in chromosome number was also observed in the three cell lines. In addition to the morphological characteristics, these cell lines displayed differential patterns of several known mammary tumor cell markers. Following xenotransplantation of the CMT cells into nude mice, DE-F and DE-SF developed tumors within 2 weeks, whereas DE-E failed to develop any visible tumor up to 8 weeks after injection. Lastly, the CMT cell lines exhibited differential chemoresistance to several anti-tumor drugs, including melatonin, cyclosporine A, tamoxifen and indole, suggesting that these cell lines can be used as a comparative experimental model for the tumorigenesis of mammary carcinomas and a valuable tool for anti-cancer drug screening.

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1. Introduction

The occurrence of spontaneous tumors in the dog provide a valid model tumor system available for testing cancer therapeutic agents or studying cancer biology; as the histopathological and biological behavior of canine tumors are similar to their counterparts in humans (MacEwen, 1990). Canine mammary tumors (CMTs) are the most common tumor type in female dogs, comprising 52% of all neoplasms (Benjamin et al., 1999; Rutteman et al., 2001). CMTs can be multiple and may vary in histology within or among different tumor sites in an individual. The tumors often have a complex morphology consisting of epithelial, mesenchymal or mixed-type cells. CMTs can be benign or malignant, with about 50% of the tumors being benign. Generally, complex adenoma and benign mixed tumor are the dominant types, whereas pure benign mesenchymal tumors are rare (Hellmén et al., 2000). Because CMTs and human mammary cancers share similar properties in biological behavior, histology and epidemiology (MacEwen, 1990; Martin et al., 1984; Misdorp and Hart, 1976; Nerurkar et al., 1989; Owen,

1979; Rutteman et al., 1988; Van Leeuwen et al., 1996; Visonneau et al., 1999; Vos et al., 1993), CMTs have been used as an important spontaneous animal model for human mammary cancer research in the past three to four decades (Kumaraguruparan et al., 2006; MacEwen, 1990; Martin et al., 1984; Pierrepoint et al., 1985; Schneider, 1970; Strandberg and Goodman, 1974; Tap et al., 1998).

The classification of canine mammary tumors is traditionally made on the basis of histological characteristics as depicted in the World Health Organization criteria for canine mammary neoplasms (Misdorp et al., 1999). Cancer is a genetic and epigenetic disease (Graham and Myers, 1999; Sarli et al., 2002). Development of mammary tumors involves aberrant accumulation of factors in cells caused by excessive expression of hormone receptors, insufficient apoptosis or dysregulation of cellular differentiation (Hanahan and Weinberg, 2000). In recent years, markers associated with the genetic or epigenetic alternations in tumor cells have become a useful tool in the categorization, invasiveness, prognosis and chemoresistance of canine and human mammary cancers (Hellmén et al., 1988, 1993; Hellmén and Lindgren, 1989). In the canine, markers such as p63, vimentin and cytokeratins have been shown to be specific to different mammary cell types. Both p63 and vimentin are markers of myoepithelial cells (Gama et al., 2003), whereas cytokeratin has been used as a sensitive marker

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for epithelial tumors of stratified and squamous cell origin (Battifora et al., 1980; Gupta et al., 1992; Ivanyi et al., 1990). For cancer malignancy, vimentin and matrix metalloproteinases (MMPs) are valuable indicators of malignancy in canine mammary tumor. It has been shown that vimentin is selectively increased in aggressive mammary cancer cell line (Gilles et al., 1999; Kokkinos et al., 2007) and that MMPs are implicated in many steps of malignancy, including primary tumor growth, angiogenesis, invasion of the basement membrane and stroma, and metastatic progression (Gilles et al., 2004). Additionally, mutations of p53 protein could serve as a useful indicator of increased malignant potential and poor prognosis in canine mammary tumors (Lee et al., 2004). Oncogene c-erbB-2 has also been shown to be correlated with the histopathological diagnosis of malignancy but not with the presence of local invasion or regional metastatic disease in canines (Ahern et al., 1996). On the contrary, metallothioneins (MTs) expression is significantly associated with the benign canine mammary tumors (Erginsoy et al., 2006). As to markers of chemoresistance, the absence of estrogen receptor (ER) is closely correlated with an increased chemoresistance in both human and canine mammary tumors (Osborne, 1999). The identification and analysis of these markers has helped tremendously to understand the mechanisms of tumorigenesis and malignancy in both human and canine mammary tumors.

In this paper we describe the establishment and characterization of three new CMT cell lines, namely DE-E, DE-F and DE-SF, derived from a single tumor mass excised from an 11-year-old female dog. These cell lines exhibited significant diversity in morphology, chromosome aberrance, marker expression, tumorigenicity and chemoresistance. Hence, these cell lines can serve as a valuable experimental model for the tumorigenesis of mammary carcinomas and as a potential tool for anti-cancer drug screening.

2. Materials and methods

2.1. Tumor specimen

An encapsulated tumor mass was surgically excised from the mammary tissues of an 11-year-old mixed breed female dog. Microscopically, the mass consisted of multiple compact nodules of neoplastic glandular or tubular cells. The extent of myoepithelial cell proliferation varied from scant to abundant in different areas of the mass. Central necrosis in the neoplastic nodules was also noted. Since the growth pattern of the tumor was multiple solitary nodules, malignant potential of the case was suspected. Overall, the histopathology examination of the tissue specimen revealed a complex mammary carcinoma.

2.2. Cell culture

The tumor specimen was washed three times in antibiotic medium (RPMI1640 with 500 IU/ml penicillin and 500 IU/ml streptomycin). The tissue was then minced with scissors and transferred to a 60-mm (in diameter) tissue culture dish (Nunc, USA), containing 10 ml of 0.25% trypsin solution (0.25% trypsin and 0.2% EDTA in PBS). The cell suspension was gently agitated with a magnetic stirrer at 4 °C for 1 h. The larger undigested tissue pieces were then allowed to settle, and the supernatant was transferred into an equal volume of complete medium (RPMI1640 with 10% fetal bovine serum FBS: Hyclone Laboratories, Logan UT; 100 IU/ml penicillin and 100 IU/ml streptomycin) and mixed well. Cells were pelleted at 1000 rpm (180g) for 5 min, and resuspended in fresh complete medium. The cells were then seeded into 25-cm² tissue culture flasks, containing 3 ml of RPMI1640 with 10% FBS, and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

2.3. Establishment of CMT cell lines

After the formation of a complete monolayer in primary culture, CMT cells were washed with PBS, treated with 0.25% trypsin solution, and incubated until cells were dislodged from the flask surface. 10⁵ cells were transferred into a new flask with fresh RPMI1640-10% FBS medium. For the first 20 subcultures, 50% culture medium was replaced with equal volume of fresh medium. The initial cultures contained several morphologically distinct cell types as observed under microscope. At passage 20, the culture was diluted in medium and seeded to a 96-well plate at the density of 1 cell/well to establish the single-cell-derived CMT lines.

2.4. Electron microscopy

CMT cells were harvested by centrifugation at 1000 rpm (180g) for 5 min. The cells were subsequently fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4 °C. The cells were then post-fixed in 1% osmium tetroxide for 2 h at 4 °C, washed in cacodylate buffer, dehydrated in graded acetone solutions and embedded in Spur's low-viscosity resin. Silver-to-gold sections were cut on a diamond knife using a Reichert-Jung Ultracut E ultramicrotome (Nussloch, Germany). Sections were stained with 2% uranyl acetate (EMS, Fort Washington, PA) in distilled water for 1 min. Ten random sections of each cell line were examined under a JEOL JEM 2000 EXII transmission electron microscope (JEOL Ltd., Tokyo, Japan). Photomicrographs were taken from a representative section of each cell line.

2.5. Growth studies

CMT cells (10⁵) at subculture 120 were seeded in 25-cm² tissue culture flasks and incubated at 37 °C. At each day for a total of 7 days, duplicate flasks of cells were trypsinized, and the cell numbers were counted with a hemocytometer. The average number of cells was calculated and recorded.

2.6. Chromosome preparation

CMT cells at subculture 120 were seeded into a 25-cm² flask at a density of 10⁵ cells/ml and grown at 37 °C in RPMI1640 medium, supplemented with 10% FBS and antibiotics. After 24 h of incubation, 0.2 g/ml of colcemid (Sigma, St. Louis, MO, USA) was added into the medium for 2 h to arrest the cells in metaphase. The flask was then gently tapped by hand to dislodge the majority of cells (90%) from the flask surface, and the cells were harvested by centrifugation 1000 rpm (180g) for 5 min. Cell pellet was resuspended in a hypotonic 0.5% KCl solution for 10 min and fixed in a 3:1 mixture of methanol:acetic acid. Slides were prepared by conventional drop-splash technique (Freshney, 1994). Finally, the cells were stained with 5% Giemsa (EMS, Washington DC, USA) for 10 min. The chromosomes were observed and counted under a Leica DMR microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

2.7. Western blot analysis

CMT cells (10⁶) were collected by centrifugation at 1000 rpm (180g) for 5 min. Prior to SDS polyacrylamide gel electrophoresis (SDS-PAGE), the cell pellets were lysed with 100 µl of sample buffer (100 mM Tris-HCl buffer, pH 6.8, 4% SDS, 0.07% β-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue). All samples were heated (100 °C, 5 min), cooled to room temperature, and subjected to 12% SDS-PAGE. The proteins were stained with Coomassie brilliant blue R-250 or electrotransferred onto a PVDF membrane (Amersham Pharmacia Biotech) for western blot analy-

Table 1

Marker Expression in DE-E, DE-F and DE-SF cells and overview of the source and dilution of the primary antibodies used.

Antibody	Source	Isotype	Clone	Dilution	Epitope	Mol. wt. of antigen	DE-E	DE-F	DE-SF
Vimentin	NeoMarkers (mouse anti-pig)	MAb IgG1	V-9	1:100	ND	57–60 kDa	+	+	+
SMA	NeoMarkers (mouse anti-human)	MAB IgG2a	1A4	1:100	Acetyl group, the first 4 aa	42 kDa	+	+	–
AE1	NeoMarkers (mouse anti-human)	MAB IgG1	AE1	1:1000	ND	40–56 kDa	+	–	–
AE3	NeoMarkers (mouse anti-human)	MAB IgG1	AE3	1:1000	ND	52–67 kDa	+	–	–
K14	Santa Cruz (mouse anti-human)	MAB IgG1	5F298	1:500	Cytoskeletal of TR146 cells	50 kDa	–	–	–
K17	Santa Cruz (rabbit anti-human)	Polyclonal	V-17	1:500	Cytokeratin 17 of human	40 kDa	–	–	–
K18	NeoMarkers (mouse anti-human)	MAB IgG1	DC10	1:200	ND	45 kDa	–	–	–
K19	NeoMarkers (mouse anti-human)	MAB IgG1	A53-B/A2.26	1:200	aa 312–335	40 kDa	–	–	–
PR	NeoMarkers (mouse anti-human)	MAB IgG2a	hPRa2+hPRa 3	1:100	N-terminal half of human PR	81 kDa (A-form) and 116 kDa (B-form)	–	–	–
ER	NeoMarkers (rabbit anti-human)	MAB rabbit IgG	SP1	1:100	C-terminus	67 kDa	–	–	–
Bcl-2	Oncogene (rabbit anti-human)	Polyclonal	Ab-2	1:20	aa 20–34	24–26 kDa	–	–	–
Bax	Oncogene (rabbit anti-human)	Polyclonal	Ab-1	1:100	aa 150–165	21 kDa	–	–	–
p53	Oncogene (mouse anti-human)	MAB IgG2a	PAb 421	1:200	aa 371–380	Wild/mutant p53	–	–	–
p63	NeoMarkers (mouse anti-human)	MAB IgG2a	4A4	1:200	aa 1–205	63 kDa	–	–	–

Smooth muscle actin: SMA; AE1: low-molecular-weight cytokeratins; AE-3: high-molecular-weight cytokeratins; keratin 14: K14; keratin 17: K17; keratin 18: K18; keratin 19: K19; PR: progesterone receptor; ER: estrogen receptor; (+): positive; (–): negative.

sis as described by Towbin et al. (1979). The membrane was washed in PBS–Tween and blocked with 5% skimmed milk overnight at 4 °C. After washing, the membrane was reacted with specific primary antibody (Table 1) for 2 h at room temperature, washed, and then hybridized with alkaline phosphatase-conjugated goat anti-mouse (Santa Cruz Biotechnology) or anti-rabbit immunoglobulin G (Santa Cruz Biotechnology). Finally, the specific protein bands were visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro-blue tetrazolium chloride (NBT). Human cancer cell lines MCF-7, MDA-MB231 and HeLa were used for comparison. MCF-7 is positive for K18, K19, PR and ER but negative for vimentin; MDA-MB231 is positive for K18, K19, vimentin and mutant p53 but negative for PR and ER; HeLa is positive for K14 and K17.

2.8. Immunocytochemistry

CMT cells were grown in Lab-Tek chamber slide™ system (four-well Permanox Slide, Nalge Nunc International Corp., IL, USA) at 37 °C overnight. After cooling the cells on ice, the culture media was removed and washed with 4 °C PBS. Then the cells were fixed in 2% para-formaldehyde/0.1% Triton X-100 for 30 min on ice. Following removal of fixative, the cells were washed twice in 4 °C PBS (5 min per wash). After washing, the CMT cells were reacted with vimentin antibody for 2 h at room temperature, washed, and then FITC-conjugated goat anti-mouse immunoglobulin G (Sigma) was added into chamber slide™ so that cells were just covered, and incubated at 4 °C for 60 min. This was washed again for four times in 4 °C PBS (5 min per wash). Finally, the specific fluoresce signals were visualized with microscope (Zeiss, Axio Observer D1, Ham-burg, Germany).

2.9. Tumor growth in nude mice

Eight-week-old athymic nude mice (BALB/c nu/nu) were used to investigate the tumorigenicity of the three CMT cell lines. A total of 1×10^7 CMT cells at passage 120 were suspended in 0.1 ml of PBS and inoculated subcutaneously into the back of each mouse. Five nude mice were tested for each cell line, respectively. Mice were observed weekly and were kept for two months. The animal study was conducted at the Academia Sinica by following its Institutional Animal Care and Use Committee Guidelines.

2.10. Susceptibility of CMT cells to anti-tumor drugs

CMT cells were seeded at a density of 5×10^4 /well into 6-well culture plates. After 24 h, the cells were treated with a panel of eight representative anti-tumor drugs follows: etoposide (100 μM) (Chiu et al., 2005; Choi et al., 2007), melatonin (10 μM) (García-Santos et al., 2006; Rubio et al., 2007), retinamide (10 μM) (Choi et al., 2004; Lytle et al., 2005), cyclosporine A (100 μM) (Beppu et al., 2003; Mongini et al., 2001), resveratrol (100 μM) (Sareen et al., 2007; Shimizu et al., 2006), tamoxifen (100 μM) (Mandlekar et al., 2000; Simard et al., 2002), indole (100 μM) (Howells et al., 2002, 2005) and doxorubicin (1 μM) (Czeczuga-Semeniuk et al., 2004; Li et al., 2007). As a control, cells were mock treated with the vehicle dimethyl sulfoxide (DMSO) or water. Three days post-treatment, cells were harvested by trypsinization, stained with 0.4% trypan blue and counted using a hemocytometer under a light microscope. At different time points post-treatment, the inhibitory effect of etoposide, resveratrol, retinamide and doxorubicin was further assayed at different concentrations: etoposide, 0, 10, 25, 50 and 100 μM; resveratrol, 0, 10, 25, 50 and 100 μM; retinamide, 0, 1, 2.5, 5 and 10 μM; doxorubicin, 0, 1, 10, 100 and 1000 nM. In the experiment, samples in triplicate of each treatment were analyzed and cell counting was conducted three times for each sample. The reported data represent the averaged cell number of triplicate samples for each treatment.

3. Results

3.1. Cell morphology

Three CMT cell lines, namely DE-E, DE-F and DE-SF were established from canine mammary carcinoma. The cells adhered well to the substratum within 2 h of plating and achieved confluence in 3 days at 37 °C after initial plating. If not subcultured, these cells would eventually overgrow into clusters. Morphologically, DE-E, DE-F and DE-SF displayed features of epithelial (Fig. 1A), fibroblast (Fig. 1B) and spindle fibroblast (Fig. 1C) cells, respectively. The ratio of cell width versus length is larger in DE-F than DE-SF under the same magnification. A common observation from 10 random sections of each cell line under transmission electron microscope demonstrated that DE-F and DE-SF cells appeared to have larger

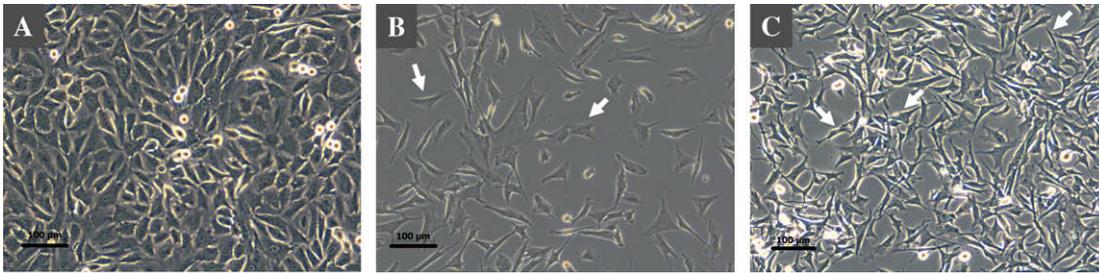


Fig. 1. Phase contrast micrograph of CMT cell lines. (A): DE-E; (B): DE-F; (C): DE-SF. Cells were observed under a phase contrast light microscope. DE-E cells displayed morphological characteristic of epithelium, whereas DE-F cells were fibroblast and DE-SF cells were spindle fibroblast. DE-SF was shorter than DE-F in length and has a smaller ratio of cell width versus length. Arrows highlight the morphological characteristics.

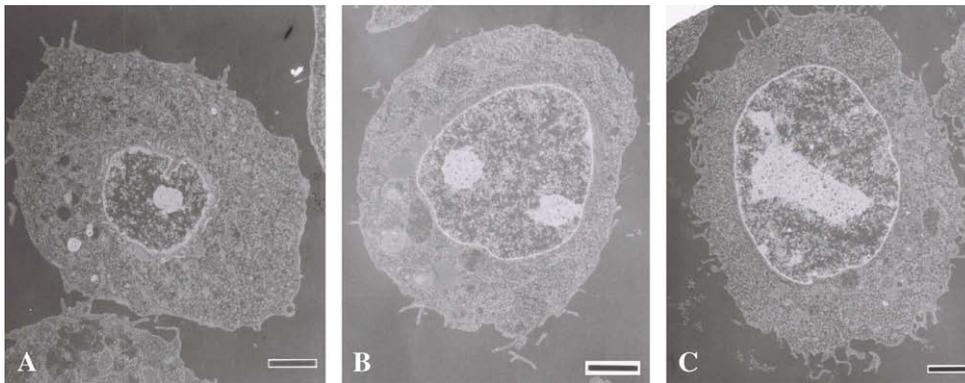


Fig. 2. Transmission electron micrographs of CMT cell lines. (A): DE-E; (B): DE-F; (C): DE-SF. Cells were fixed in 1% osmium tetroxide and embedded in Spur's low-viscosity resin. Ultramicrotomed sections were stained with 2% uranyl acetate and examined under a JEOL JEM 2000 EXII transmission electron microscope (JEOL Ltd., Tokyo, Japan). Bar = 2 μ m.

nucleoli and a higher nuclear/cytoplasmic ratio than DE-E did (Fig. 2).

3.2. Subculture, growth and storage

All the three CMT cell lines were initially cultured in RPMI1640 medium supplemented with 10% FBS. During the first 10 passages, a conditioned medium containing 50% each of the new and old medium was used to subculture the CMT cells at an 8-day interval. Subsequent subcultures 11–40 and 41–80 were made at 6- and 3-day intervals, respectively, in fresh RPMI1640–10% FBS medium. After 100 passages, the amount of FBS in the medium was reduced to 5% for all three cell lines. Contact inhibition was observed in all three cell lines up to 50 passages, gradually diminishing, and finally disappeared after passage 100 (data not shown). Stocks of these CMT cell lines have been stored in liquid nitrogen every 10 passages since establishment. Cells recovered from the frozen stocks after 1 year showed 90% viability and could grow to confluence in 2 days. By the time when this manuscript was being prepared, these CMT cell lines have been grown in culture for over 200 passages. The latest doubling time of cell population was estimated to be approximately 30 h for all three CMT cell lines (Fig. 3).

3.3. Chromosome analysis

The number of chromosomes in DE-E, DE-F and DE-SF cells ranged from 60 to 148, 32 to 90 and 50 to 85, respectively (Fig. 4). The major distribution of chromosome number in these cells was 114 and 115 in DE-E (Fig. 4A); 56, 64 and 74 in DE-F (Fig. 4B); and 78 and 79 in DE-SF (Fig. 4C)

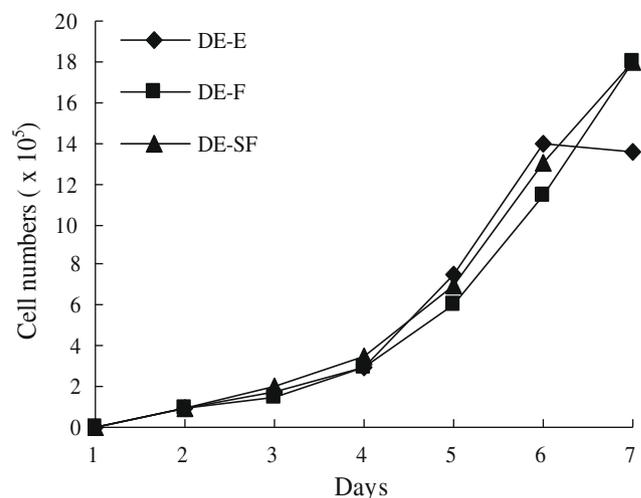


Fig. 3. Growth curve of DE-E, DE-F and DE-SF cell lines. 10^5 cells were seeded in 25-cm² tissue culture flasks and incubated at 37 °C. At different time points after seeding, cells were trypsinized and the number of cells was measured with a hemocytometer under Leica DMR microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). The average of duplicate samples at each time point was calculated and shown in the figure.

3.4. Cellular protein expression profiles

The expression profile of tumor-related markers was assessed by western blot analysis to characterize the three CMT cell lines. In the assay, equal amount of proteins from each cell line was loaded onto a SDS polyacrylamide gel (Fig. 5A). Differential protein banding patterns was observed in the three CMT cell lines, indicat-

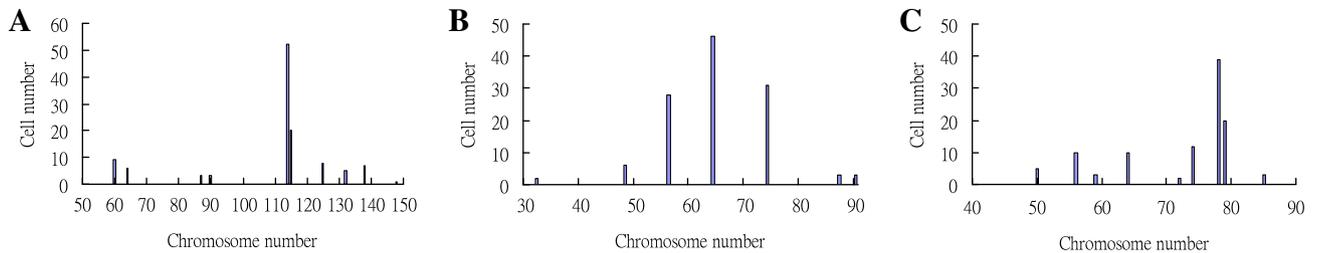


Fig. 4. Frequency distribution of chromosome numbers of CMT cell lines. (A): DE-E; (B): DE-F; (C): DE-SF. Cells arrested in the metaphase with colcemid were subjected to hypotonic treatment with 0.5% KCl, and were fixed in a 3:1 mixture of methanol:acetic acid. The spreading of chromosomes was prepared on a glass slide by using the drop-splash technique, followed by staining with 5% Giemsa. The numbers of chromosomes were counted under a Leica DMR microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

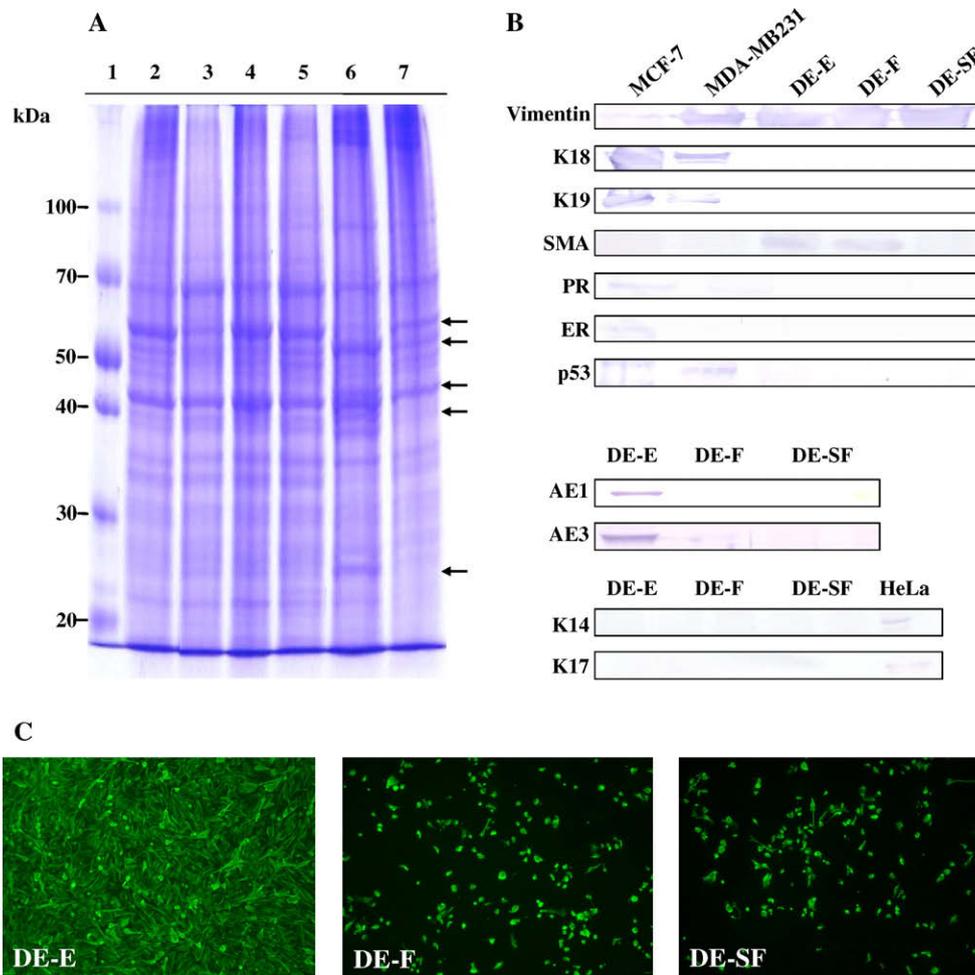


Fig. 5. Expression of cell markers in DE-E, DE-F and DE-SF cell lines. Equal amount of proteins from each cell line was loaded onto a SDS polyacrylamide gel for analysis (A). In panel A: lane 1, pre-stained protein marker; lane 2, HeLa; lane 3, MCF-7; lane 4, MDA-MB231; lane 5, DE-E; lane 6, DE-F; lane 7, DE-SF. The gel was stained with Coomassie brilliant blue R-250. Arrows highlight the differential protein patterns observed in the three CMT cell lines. The expression of vimentin, keratins (K14, K17, K18, K19), smooth muscle actin (SMA), progesterone receptor (PR), estrogen receptor (ER), p53, low-molecular-weight cytokeratin (AE1), and high-molecular-weight cytokeratin (AE3) in DE-E, DE-F and DE-SF cell lines was analyzed by western blotting with specific antisera (B). The expression of vimentin in DE-E, DE-F and DE-SF was further analyzed by immunohistochemistry (C). Human cancer cell lines MCF-7, MDA-MB231 and HeLa were used for comparison. MCF-7 is positive for K18, K19, PR and ER; MDA-MB231 is positive for vimentin and p53; HeLa is positive for K14 and K17.

ing differences in entity of the three cell lines. The selected markers for analysis were cytokeratins AE1 (low-molecular-weight) and AE3 (high-molecular-weight), smooth muscle actin (SMA), keratins (K14, K17, K18, K19), p63, mesenchymal marker (vimentin), breast cancer related steroid hormone receptors progesterone receptor (PR) and ER, and apoptosis-associated genes (p53, Bax and Bcl-2). As shown in Fig. 5, the expression of vimentin protein in all three cell lines was confirmed by western blot as well as immunohisto-

chemical staining (Fig. 5B and C), whereas the expression of AE1 and AE3 cytokeratins were detected only in DE-E cells. By western blot, the expression of SMA was detected in DE-E and DE-F cells (Fig. 5B), whereas the expression of p63, K14, K17, K18, K19, PR and ER were not detectable in all three cell lines (Fig. 5B and Table 1). Lastly, the apoptosis-associated proteins (p53, Bax and Bcl-2) were not detected by western blot in all three cell lines (p53 in Fig. 5B; Bax & Bcl-2, data not shown).

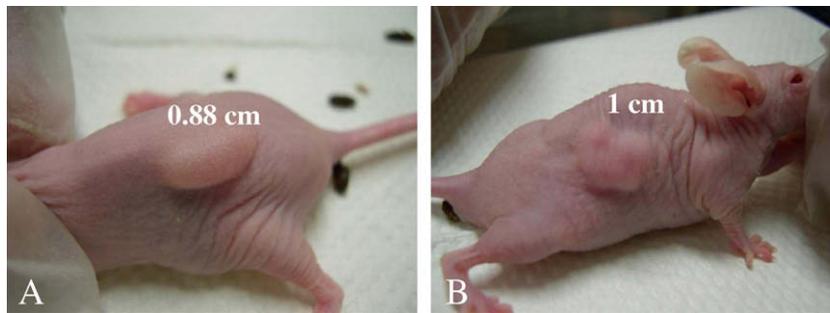


Fig. 6. Tumorigenesis of DE-E, DE-F and DE-SF cells in nude mice. Each cell line at passage 120 was injected subcutaneously into five eight-week old athymic mice (BALB/c nu/nu), respectively. Mice were observed weekly for two months. The size of tumor developed in the DE-F (A) and DE-SF (B) injected mice at 8 weeks post-injection is depicted here. No visible tumor mass was found in the DE-E injected mice up to 8 weeks post-injection (data not shown).

3.5. Tumorigenesis by xenotransplantation

To examine the tumorigenicity of the three CMT cell lines, each cell line at passage 120 was injected subcutaneously into five eight-week-old athymic mice (BALB/c nu/nu), respectively. Tumor mass at the injection site was first observed in all five mice injected with DE-SF cells at one week after injection and the size of the mass reached 0.86–1 cm in diameter at week 8 (Fig. 6B). At week 2 post-injection, tumor mass in all five mice injected with DE-F became noticeable and grew to the size of 0.62–0.88 cm in diameter at week 8 (Fig. 6A). In addition, the mass on DE-SF-injected mice grew firm as examined by palpation at week 8, whereas the mass on DE-F-injected mice were relatively soft. No tumor mass was observed in the DE-E injected mice up to 8 weeks post-injection (data not shown).

3.6. Susceptibility of CMT cells to anti-tumor drugs

The susceptibility of the three CMT cell lines to eight representative anti-tumor drugs was first characterized at their common effective concentrations reported for *in vitro* assays. As shown in Table 2, all three CMT cell lines were resistant to melatonin, cyclosporine A, tamoxifen and indole; whereas etoposide, resveratrol, retinamide and doxorubicin effectively inhibited the growth of all three cell lines. Microscopically, all three CMT cell lines exhibited dramatic morphological changes characterized as cell rounding, followed by cell detachment by 24 h after treatment with 10 μ M etoposide, 25 μ M resveratrol and 2.5 μ M retinamide, respectively. With doxorubicin treatment, DE-F and DE-SF exhibited dramatic morphological changes characterized as cell rounding at 10 nM, whereas DE-E cells were resistant to the drug up to 1 μ M.

The inhibitory effect of etoposide, resveratrol, retinamide and doxorubicin was further characterized by a time-course study with the drugs at different concentrations. As shown in Table 3, etopo-

side, resveratrol and retinamide inhibited the CMT cells in a dose-dependent manner. As determined at 72 h after drug treatment, the dose causing 50% cell death of the CMT cells is as follows: etoposide (μ M) – 5.7 (DE-E), 5.2 (DE-F), 5.2 (DE-SF); resveratrol (μ M)– 35.6 (DE-E), 67.9 (DE-F), 48.6 (DE-SF); retinamide (μ M) – 3.5 (DE-E), 5.3 (DE-F), 4.9 (DE-SF). For doxorubicin, treatment with 10 nM doxorubicin resulted in a loss of approximately 47% and 65% in DE-F and DE-SF cells; death of DE-E cells was only observed at a much higher concentration (i.e. 1 μ M).

4. Discussion

In this manuscript, we report the establishment and characterization of three CMT cell lines derived from one single malignant mammary tumor mass in a female dog. Despite the same anatomical origin, these cell lines exhibited diverse characteristics in morphology and tumorigenesis. Morphologically, DE-E, DE-F and DE-SF were characterized as epithelia, fibroblast and spindle fibroblast, respectively. In addition, examination by electron microscopy revealed that both DE-F and DE-SF appeared to have a high nucleus/cytoplasm ratio, a phenomenon often associated with malignant behavior of tumor cells. Indeed, when injected into athymic nude mice, both DE-F and DE-SF developed prominent tumor masses in the injected mice within 2 weeks, whereas DE-E failed to develop any visible tumor up to 8 weeks after injection. Nonetheless, the possibility can not be ruled out that the differential morphological characteristics were an artifact introduced during sample preparation for electron microscopy as the differences were not as clear when cells were observed under light microscope (Fig. 1).

The human and canine mammary gland ductal and lobular system consists of two major epithelial cell types, i.e., luminal and myoepithelial cells. In both human and canine, luminal cells are characterized by expression of K7, K8, K18, K19 and epithelial membrane antigen (EMA), whereas the majority of myoepithelial cells express vimentin, K14, K17, p63, and SMA (Abd El-Rehim et al., 2004; Gama et al., 2003; Mark et al., 1990; Saraiva et al., 2008). Although all three CMT cell lines expressed vimentin, they did not express K14, K17, or p63. Nonetheless, both DE-E and DE-F cell lines did express SMA. As to luminal cell markers, we have tested only K18 and K19 and found none of the three cell lines expressed either K18 or K19. Interestingly, DE-F cells also express cytokeratin AE1 and AE3, which generally present in the glandular cells.

In adult human and canine mammary tissues, vimentin is regarded as not only a hallmark of myoepithelial cell but also an indicator of the end-stage of tumor cell dedifferentiation. Vimentin-expressing cells are rarely found in invasive breast cancers, which predominantly derived from the glandular cells (Korsching et al.,

Table 2
Chemoresistance^a of DE-E, DE-F and DE-SF cells.

Anti-cancer drugs	DE-E	DE-F	DE-SF
Etoposide (100 μ M)	S	S	S
Melatonin (10 μ M)	R	R	R
Retinamide (10 μ M)	S	S	S
Cyclosporin A (100 μ M)	R	R	R
Resveratrol (100 μ M)	S	S	S
Tamoxifen (100 μ M)	R	R	R
Indole (100 μ M)	R	R	R
Doxorubicin (1 μ M)	R	S	S

^a Test conducted at reported common effective doses. Cell response is defined as sensitive (S) or resistant (R) at an arbitrary cut-off of 5% cell loss after treatment.

Table 3
Inhibitory effects of etoposide, resveratrol, retinamide and doxorubicin on the CMT cells.

	Etoposide (μM)					Resveratrol (μM)					Retinamide (μM)					Doxorubicin (μM)					
	0	10	25	50	100	0	10	25	50	100	0	1	2.5	5	10	0	1	10	100	1000	
<i>DE-E</i>																					
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
24 h	4.8	3.2	2.9	1.8	1.7	4.8	5.1	3.8	3.8	3.2	4.8	4.5	4.2	3.5	3.2	4.8	5.1	4.7	4.3	4.6	4.6
48 h	11.6	3.3	2.7	1.5	0.9	11.6	9.3	7.2	2.9	2	11.6	8.3	7.2	2	2	11.6	11.5	11	10.5	10	10
72 h	20.9	2.7	2.1	0.8	0.6	20.9	21.2	16.7	6.2	2.6	20.9	19.7	13.4	2.7	2.6	20.9	20	20.2	19	17	17
<i>DE-F</i>																					
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
24 h	15.3	6.2	4.5	3.9	3.9	15.3	8.4	7.5	6.5	5.6	15.3	15	12.6	11.1	10.5	15.3	15.2	12.1	3	2	2
48 h	24.8	2.4	1.5	1.5	0.9	24.8	15	13.2	8	7.2	24.8	24.6	14.6	11.1	4.4	24.8	24.8	15.6	1	0.8	0.8
72 h	34.2	1.4	0.8	0.8	0.3	34.2	33.9	25.7	21.6	6.8	34.2	34.5	25.8	18	3.3	34.2	33.9	18	0.3	0	0
<i>DE-SF</i>																					
0	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
24 h	16	6	4	3	4	16	8.4	8	6.7	6	16	16	13	11.1	8	16	15	10.8	7.1	3.5	3.5
48 h	26	3	2	2	0.5	26	18	14	8	6.5	37	36	23	18	3	26	24	12.1	5.7	1.5	1.5
72 h	37	1.2	0.6	0.5	0.3	37	33.9	26	18	6	37	36	23	18	3	37	33	12.9	0.8	0	0

2005). Although rare, vimentin-positive mammary tumors are mostly malignant and highly invasive. Yet, the origin of vimentin-positive tumor cells is uncertain at this moment (Chen et al., 2008; Domagala et al., 1990; Hellmén et al., 2000; Hendrix et al., 1997; Kokkinos et al., 2007; Patricia et al., 1999). There is growing evidence of the existence of vimentin-positive progenitor cells with a putative bilinear differentiation potential or a potential towards myoepithelial cells in normal human and mouse breasts (Clayton et al., 2004; Li et al., 2003; Pechoux et al., 1999). Based on recent studies, a hypothesis has been proposed that vimentin-expressing mammary carcinomas might derive from progenitor cells with a bilinear differentiation potential into glandular and myoepithelial cells (Korsching et al., 2005). This hypothesis is further supported by the finding of vimentin expression in the adjacent ductal carcinoma in situ components, a phenomenon contradicting the idea that vimentin expression is a late event in carcinogenesis after tumor invasion. Based on the hypotheses, the three CMT cell lines could have derived from different tumor cell subpopulations in the same tumor mass. Alternately, these cell lines might represent different stages of dedifferentiation of a single tumor population. It is of great interest to illustrate the origin and development of the three CMT cell lines in future study.

All three CMT cell lines did not express either ER or PR. Both estrogen and progesterone have been shown to play an important role during development of mammary tumors. While both ER and PR are expressed at relatively high level in canine normal mammary tissue, the level of both receptors drops in malignant cases (Rutteman et al., 1988). Sartin et al. (1992) showed that an absence of ER or PR was associated with shorter survival in canine victims of mammary cancer. In the case of ER, metastases of canine mammary tumors often fail to express the receptor (Kesse-Adu and Shousha, 2004; Sheikh et al., 1994). In human mammary cancer, ER expression is present in about 70% of the cases; patients with ER-positive/PR-positive tumors have a better prognosis than those with ER-negative/PR-negative tumors (Gown, 2008; Williams et al., 2009). The absence of ER in human and canine mammary tumor cells has also been shown to be correlated with an increased resistance against some frequently used chemotherapeutic agents, such as the anti-estrogen drug tamoxifen (Osborne, 1999). Our data also demonstrated that the three CMT cell lines were ER-negative and highly resistant to the tamoxifen treatment. p53, Bcl-2 and Bax are major regulators involved in cell growth and apoptosis, and have been used as prognostic markers for mammary cancers because of their crucial roles in tumor progression and susceptibility to anti-cancer agents (Coradini and Daidone, 2004; Daidone et al., 1999; Elledge and Allred, 1998; Jager et al., 2002; Krajewski

et al., 1999; Porebska et al., 2006). p53 can activate the pro-apoptotic protein Bax by binding to the anti-apoptotic proteins Bcl-XL and Bcl-2, resulting in mitochondrial membrane permeabilization and induction of apoptosis (Vousden, 2000). Many studies have shown that mutation of the p53 gene is the most common genetic alteration in canine mammary tumors as well as in other human and canine malignancies. Because of a very short half-life, the wild-type p53 protein is normally not detectable in cells. On the contrary, the mutant p53 proteins are relatively stable, resulting in the accumulation of the proteins in tumor cells to a level that can be detected by immunological methods. Therefore, the presence of detectable p53 protein is regarded as a marker of tumor cells (Rodrigues et al., 1990; Rotter, 1983). None of the three CMT cell lines expressed p53, Bcl-2 or Bax protein in the amount detectable by the western blotting in this study. Because the antibodies used in the study are capable of detecting the normal canine p53 and its mutants, the data indicate that p53 mutation may not be a factor of the tumorigenesis of these three CMT cell lines. Nonetheless, further investigation, such as sequencing of the p53 gene in these cells, is required to verify the hypothesis.

In summary, we have established three new CMT cell lines originated from the same malignant breast tumor mass. DE-E is epithelial in morphology, non-tumorigenic in nude mice, and is characterized as vimentin⁺/AE1⁺/AE3⁺/SMA⁺; DE-F is fibroblast, tumorigenic in nude mice, and have an expression profile of vimentin⁺/SMA⁺; DE-SF is spindle fibroblast and tumorigenic in nude mice, with an expression profile of vimentin⁺. These cells are resistant to anti-tumor drugs melatonin, cyclosporine A, tamoxifen and indole. In addition, they display differential susceptibility to etoposide, resveratrol, retinamide and doxorubicin. During the development of a tumor, the millions of same-mother-cell-derived cells that make up a detectable lump become highly diversified, a phenomenon known as tumor heterogeneity. The three CMT cell lines might represent a glimpse of the heterogeneous cell populations in a canine breast cancer mass as they display different characteristics in morphology and marker expression. Because of their differential tumorigenicity and chemoresistance, these cell lines can be used as a valuable experimental model for comparative study of the complex relationship among tumor dedifferentiation, tumorigenesis and chemoresistance of mammary tumors. The finding of resistance to multiple anti-cancer drugs in the non-tumorigenic DE-E is interesting. Part of the resistance exhibited by DE-E could be attributed to the aberrant expression of cell receptor, for example, the lack of ER resulting in the resistance to tamoxifen. Nonetheless the enhanced resistance of DE-E cells to doxorubicin is probably the most intriguing finding. Doxorubicin

is an anti-cancer drug used to treat many types of cancers, including leukemias, various carcinomas and soft tissue sarcomas. Doxorubicin prevents DNA replication via acting through intercalating double-stranded DNA and immobilizing the topoisomerase II complex (Fornari et al., 1994). The mechanism underlying the resistance to doxorubicin in DE-E cells is unknown. However, it has most recently been found that microRNAs (miRNAs) might play a crucial role in the resistance to doxorubicin in human breast cancer MCF-7 cells (Chen et al., 2009). DE-E cells thus can be used as a comparative system in future investigation on the role of miRNAs in the drug resistance of mammary tumors.

Acknowledgement

This study was supported by Grant No. NSC-95-2622-B-197-002-CC3 from the National Science Council, Taiwan.

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