Establishment and Characterization of Eight Feline Mammary Adenocarcinoma Cell Lines

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ABSTRACT. Eight new feline mammary adenocarcinoma cell lines derived from either primary or metastatic lesions were established. The morphology of all the cell lines was epithelioid and round to spindle in shape, with cell growth occurring in a monolayer fashion. On immunohistochemistry, these cells reacted with anti-keratin and anti-vimentin antisera. The doubling time of these cells was between 19 and 54 hr. Tumor masses were developed in nude mice by subcutaneous inoculation of the cells that were histologically identical to their original mammary tumor lesions. Telomerase activities measured using the telomeric repeat amplification protocol assay revealed high telomeric activity in all of the cells.

KEY WORDS: feline, mammary tumor cell line, primary and metastatic lesion.


Feline mammary cancer (FMC) is the third most common neoplasm following hematopoietic and skin tumors [1, 6]. Most cases of FMC are histologically adenocarcinoma [3]. This tumor shows local infiltrative-destructive growth and frequently metastasizes to the regional lymph nodes, lung and pleura in the early stages of disease [6, 15]. Establishment of cell lines from FMC has been conducted, however the number of established cells has been limited [2, 8, 10], suggesting that the establishment of FMC cell lines is difficult compared to those of other animal species. FMC has many features, including specific biological behaviors and histopathological appearance as well as poor prognosis, and may be an attractive model for research on human breast cancer [7, 9, 15]. We report herein the establishment and characterization of eight FMC cells derived from either primary or metastatic lesions of five cats with spontaneous FMC.

Tissue samples were obtained from 13 cats with FMC admitted to the Veterinary Hospital at the University of Tokyo (Fig. 1). Tumor cells were collected from the surgical specimen or by thoracocentesis in the cats with thoracic metastasis. Tissue samples were placed in 50-mL tubes with phosphate buffer solution (PBS) supplemented with 0.2-mg/mL gentamycin sulfate (Sigma Chemical Co., St. Louis, MO, U.S.A.) and kept overnight at 4°C. They were then minced into 1- to 2-mm3 pieces and digested with collagenase mixed solution of DNase and pronase (Sigma Chemical Co.) for 1 hr at 37°C under constant stirring. The digested cells were then incubated in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 20% fetal bovine serum (FBS) (Equitech-Bio Inc., Ingram, TX, U.S.A.), 0.01 mg/mL L-glutamine (Nissui Pharmaceutical Co.), fungizone (Gibco BRL., Grand Island, N.Y., U.S.A.) and 5 mg/mL gentamycin sulfate (Sigma Chemical Co.), and incubated at 37°C in a humidified atmosphere of 5% CO2. Cells collected from pleural effusion were centrifuged and washed with PBS, then cultured under the same conditions described above. After the 10th passages, when cell growth seemed to be stable, the concentrations of FBS in the culture medium were decreased from 20% to 10%.

Eight feline mammary adenocarcinoma cell lines from 5 of 13 cats were successfully established and characterized. The breed, age, gender, tissue source, histological type and...
clinical staging [11] of these patients are summarized in Table 1. These established cells, designated as FYMp, FKNp, FONp, FMCp1, FMCp2, FONm, FMCm and FNNm, showed stable proliferation over 90 passages in culture adopting a paving stone arrangement. Of those cells, FYMp, FKNp, FONp, FMCp1, FMCp2 were established from the primary lesion, while the other 3 were established from the metastatic lesions.

For light microscopy, the cells grown on cover slips within the flask were fixed in methanol and stained with Giemza solution. The cells were epithelioid and round to spindle in shape and had large nuclei and often two or more nucleoli (Fig. 2). There were multiple vacuoles in the cytoplasm. In the culture, cells formed tightly packed monolayer colonies and often adenose organoid structures. Ultrastructural studies revealed the morphology of these cell lines with large irregular nuclear outlines, some vacuole structures, numerous free ribosomes, mitochondria, endoplasmic reticulum and tonofilament bundles which were found within epithelial cells. The cell surface exhibited numerous microvilli (Fig. 3). The morphological findings of all cell lines were similar.

An indirect immunofluorescence assay was performed to detect the expression of keratin and vimentin using rabbit polyclonal anti-keratin (DAKO, Carpinteria, CA, U.S.A.) and goat anti-vimentin (ICN Biomedicals, Inc., CostaMesa, OH, U.S.A.) antibodies. Cells were then stained with fluorescein-conjugated anti-rabbit IgG (ICN Biomedicals, Inc.) or FITC-anti-goat IgG (Cappel, Aurora, OH, U.S.A.) as a second antibody. All the cultured cells examined were positively stained with anti-vimentin and anti-keratin antibodies.

The doubling time (DT) was measured as follows. Cells of 50th passages were plated in 24-well plates (Sumitomo Bakelite, Tokyo, Japan) at a concentration of $1 \times 10^5$/ml and cultured in RPMI 1640 supplemented with 10% FBS. The number of viable cells was determined every 24 hr, and the doubling time at a logarithmic phase was calculated. As a result, DTs of FYMp, FKNp, FONp, FMCp1, FMCp2,

![Table 1. Cats with mammary tumors used in this study](image)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Source of the tumor cells</th>
<th>Method of collection</th>
<th>Clinical Stage TMN</th>
<th>Histopathological diagnosis</th>
<th>Number of cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>JD a)</td>
<td>15y</td>
<td>female</td>
<td>Primary mass</td>
<td>Surgery</td>
<td>T3N2(+)M0 Stage III</td>
<td>AC c) FYMp</td>
<td></td>
</tr>
<tr>
<td>Persian</td>
<td>12y</td>
<td>female</td>
<td>Primary mass</td>
<td>Surgery</td>
<td>T1bN1(+)M0 Stage II</td>
<td>AC FKNp</td>
<td></td>
</tr>
<tr>
<td>Siamese</td>
<td>12y</td>
<td>female</td>
<td>Primary mass, Metastasis</td>
<td>Surgery</td>
<td>T3cN1(+)M0 Stage III</td>
<td>AC FONp</td>
<td></td>
</tr>
<tr>
<td>JD</td>
<td>12y</td>
<td>female</td>
<td>Primary mass, Metastasis</td>
<td>Thoracocentesis</td>
<td>T1bN0(–)M1 Stage IV</td>
<td>AC FMCp1, FMCp2</td>
<td></td>
</tr>
<tr>
<td>Siamese</td>
<td>12y</td>
<td>spayed female</td>
<td>Metastatic pleural effusion</td>
<td>Thoracocentesis</td>
<td>T1bN0(–)M1 Stage IV</td>
<td>AC FMCm, FNNm</td>
<td></td>
</tr>
</tbody>
</table>

a) JD: Japanese domestic cat. b) RLN: Regional lymph node. c) AC: Adenocarcinoma.

![Fig. 2. Light microscopy of cell lines (Giemsa stain × 1,500). Each cell line shows round to spindle-shaped cells.](image)

![Fig. 3. Transmission electron micrograph of the FNNm cell at passage 37 (× 2,500). Tonofilament bundles were observed in the cytoplasm (× 10,000).](image)
Table 2. Doubling time and telomerase activity of cell lines derived from spontaneous feline mammary adenocarcinomas

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (hr)</th>
<th>Telomerase activities ($\mu$g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FYMp</td>
<td>24.0</td>
<td>298.0</td>
</tr>
<tr>
<td>FKnp</td>
<td>36.6</td>
<td>52.9</td>
</tr>
<tr>
<td>FONp</td>
<td>29.4</td>
<td>204.0</td>
</tr>
<tr>
<td>FMCp1</td>
<td>37.9</td>
<td>158.4</td>
</tr>
<tr>
<td>FMCp2</td>
<td>41.9</td>
<td>878.4</td>
</tr>
<tr>
<td>FONm</td>
<td>54.0</td>
<td>179.7</td>
</tr>
<tr>
<td>FMCm</td>
<td>29.7</td>
<td>153.5</td>
</tr>
<tr>
<td>FNNm</td>
<td>19.1</td>
<td>425.0</td>
</tr>
</tbody>
</table>

FONm, FMCm and FNNm were 24.0, 36.6, 29.4, 37.9, 41.9, 54.0, 29.7 and 19.1 hr, respectively (Table 2).

The protocol of tumor cell transplantation into nude mice was approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo, and was performed as follows. Five-week-old female BALB/c nu/nu mice (Nippon SLC, Hamamatsu, Japan) were irradiated with a dose of 4 Gy of X-ray. After 3 days of irradiation, the suspension of $1 \times 10^7$ cells in 0.3 ml PBS was transplanted subcutaneously into the lateroventral area of nude mice. All mice were euthanized when tumors reached a diameter of 2 cm or after 12 months of transplantation when a palpable tumor mass was not detected. Tumor masses and the organs (the swollen lymph nodes, lung, liver, spleen, kidney and heart) were removed and fixed in 10% neutral buffered formalin, and then stained with hematoxylin and eosin for histopathology. Tumor growth at the transplanted sites in the nude mice was recognized in most of the cells except for FONm. All mice developing tumor masses showed metastasis to the axillary and inguinal lymph nodes. In the mice transplanted with FYMp and FMCp1 cells, lung metastasis was also recognized. Lung and liver metastasis was observed in the mice transplanted with FNNm cells. Histologically, these masses showed features similar to the primary lesions.

Telomerase activity was measured by telomeric repeat amplification protocol (TRAP) assay to confirm sufficient telomeric length, which is the index of the property of unlimited growth potential. The assay was performed using the TRAPEze telomerase detection kit (Oncor, Gaithersburg, MD, U.S.A.) with a sample of extract from 2 $\times 10^7$ cells for each assay. The 5'-end of the TS primer was labeled with $\gamma$-32p-ATP (Amersham, Buckinghamshire, England) and T4 polynucleotide kinase (Toyobo, Osaka, Japan). A 2-$\mu$l sample of the cell extract was incubated in 50 $\mu$l of reaction mixture supplied by the manufacturer containing dNTPs, TS primer, RP primer, K1 primer and TSK1 template (for amplification of an internal control), and Taq polymerase (Takara, Kyoto, Japan); this mixture was subjected to 27 cycles of polymerase chain reaction (PCR). Telomerase activity was quantified by comparing the densitometric intensity of the signals (Bio-Rad Laboratories, Hercules, CA, U.S.A.). As a result, telomerase activity was detected in all the cell lines, with the activities ranging from 52–878 U/5 $\times$ 10^3 cells (Table 2).

The first FMC cell line was established from 1 of 30 FMC patients [10]. Elshire has reported a failure to establish FMC cell lines from 14 mammary carcinomas [2]. Minke et al. have reported establishing 4 cell lines from 135 FMC patients [8]. Their histological appearance and growth pattern were similar to those of FMC cell lines established in this study [2, 10]. The establishment of FMC cell lines seems to be very difficult. The reason for this difficulty in establishing FMC cells remains unknown. In the present study, 8 cell lines were established from 5 of 13 cats with mammary carcinoma; therefore, the success rate for establishment was quite high. Tissues obtained from cats were placed in 50-ml tubes with PBS supplemented with a high dose of antibiotic and left overnight at 4°C for the purpose of eliminating infectious agents, secretion and blood from the mammary tumor glands, which possibly enables the release of inflammatory cells accumulated within tumor tissues. In addition, the procedure also eliminated the toxic factors released from apoptotic cells. These procedures may contribute to the high success rate of establishment in this study. The high success rate may also be attributed to the use of 20% FBS until cells showed stable growth in the in vitro environment.

The eight established cell lines were from either primary or metastatic lesions of spontaneous feline mammary adenocarcinoma patients. Among them, FONp and FONm as well as FMCp1, FMCp2 and FMCm were established from primary and metastatic lesions of the same cat, respectively. All the cell lines showed intense immunoreactivity for keratin, which is known as a hallmark of epithelial cells [4] and also for vimentin, which is known to be an intermediate filament of mesenchymal cells in vivo [14]. Vimentin expression may be associated with a loss of cell-to-cell contact [12], and it has been suggested to be a common protein of incohesive cells [13]. Some investigators have reported an association between detection of vimentin and a high histological grade [12].

The properties of the eight cell lines are typical for epithelial cell cultures based on the growth patterns on plastic, the cellular morphology, as observed by light microscopy and ultrastructural features. The growth patterns in the medium were similar between the wells from primary and metastatic lesions. All of the cell lines formed a number of small colonies and multiplied with tight cell-cell adhesion. There were no obvious differences among the eight cell lines in morphology, although the cell sizes among the lines were slightly different.

Telomerase is a ribonucleoprotein that synthesizes telomere repeats. Telomeres are located at chromosome ends and are reduced in length during DNA replication. Tumor cells are considered to possess unlimited growth potential, which may be an effect of their highly active telomerase, which maintains the telomeres [5]. In all of the established cell lines in this study, telomerases were present in the TRAP
assay, suggesting that all of the cells had tumorigenic properties.

In conclusion, we established 8 FMC cell lines though we did not perform the cloning of these cell lines in this study. Cloning of these cell lines may reveal features of FMC cells in detail. Since these established FMC cells maintained their original histologic features and had tumorigenic properties, they were considered to be very useful tools for further investigation of the biology and metastases of mammary tumors in cats and humans.

REFERENCES