Establishment of an immortalized cell line and transplantable xenograft from a bronchioloalveolar lung carcinoma of a cat

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Objective—To establish an immortalized cell line and transplantable xenograft of feline bronchioloalveolar lung carcinoma (BAC).

Sample Population—Pleural effusion from a 12-year-old Persian male cat with BAC.

Procedure—Tumor cells from the pleural effusion were grown in monolayer cell culture and injected into severe combined immunodeficient (SCID) mice to establish an immortalized cell line as well as a transplantable xenograft.

Results—Both the primary lung carcinoma, the derived cell line, and the transplantable xenograft had evidence of a type-II pneumocyte origin expressing lamellar bodies ultrastructurally and thyroid transcription factor-1 and surfactant immunocytochemically. All 3 also expressed nuclear p53 immunoreactivity. A metaphase spread of the cell line (SPARKY) probed with fluorescein-labeled genomic feline DNA gave evidence of its feline origin. Flow cytometric studies indicated aneuploidy with a DNA index of 1.6. An R-band ed karyotype revealed a modal number of 66 including the feline Y chromosome. The cell line had a doubling time of 16 hours. The xenograft (SPARKY-X) reached a diameter of 1 cm in 3 weeks in SCID mice. Deoxyribonucleic acid fingerprint analysis revealed that SPARKY and SPARKY-X were novel and strongly matched each other, except for the murine component found in SPARKY-X. Interestingly, SPARKY-X manifested the characteristic lepidic growth pattern of pulmonic BAC.

Conclusions—Both the cell line and xenograft retained their autochthonous BAC phenotype, making them useful for the subsequent dissection of molecular abnormalities in feline BAC and in vitro screening of chemotherapeutic agents. (Am J Vet Res 2002; 63:1745–1753)

Despite the prevalence of solid cancers in small animals, there is a paucity of established cell lines and transplantable xenografts from which to advance our knowledge of the molecular and biological properties of these tumors. This is especially true for lung cancer. Lung cancer occurs in dogs and cats where it is observed most commonly in purebred and geriatric animals. Of the types of lung cancer, adenocarcinoma is the most common. Bronchioloalveolar lung carcinoma (BAC) is a subtype of adenocarcinoma characterized by a tumoral growth pattern where the tumor cells grow along alveolar spaces preserving the native architecture of the lung. This pattern is often described as lepidic, the Greek word for scales, because the pattern that is outlined in the lung resembles the scales of a fish or butterfly wing. Bronchioloalveolar lung carcinoma is a common cancer in humans and sheep. In all of the species that it affects, BAC is a disease involving the malignant transformation of the Type-II pneumocyte, the cell that lines the alveolar spaces and secretes surfactant. The disease can develop as either solitary multicentric nodules in 1 or several lobes, including bilateral involvement, or as a diffuse pneumonic process producing lobar consolidation. The cause of disease in humans and small animals remains undefined; the cause of the disease in sheep is a slowly transforming retrovirus called the jaagsiekte retrovirus. Bronchioloalveolar lung carcinoma in humans has a weak noncausal association with smoking. In sheep and small animals (dogs and cats), the disease often is identified in the late stage with pleural effusions. Although early stage BAC can be treated effectively with surgery, there is no good treatment for late-stage BAC in animals or humans because the disease is resistant to radiation therapy and chemotherapy.

The reasons for the paucity of cell lines that have been established from small-animal carcinomas, especially lung cancers, are not clear. The literature to date contains no reports of the establishment of a cell line and transplantable xenograft from feline BAC. The successful establishment of such a cell line and xenograft would hence be important, because it would allow us to advance our knowledge of the molecular and biological properties of these tumors. The purpose of the study presented here was to establish the first immortalized cell line and transplantable xenograft of feline BAC.

Materials and Methods

Cat with BAC—A 12-year-old Persian cat (named Sparky) with shortness of breath was found to have bilateral xanthochromic pleural effusions. Histologic evaluation of fluid obtained by pleuracentesis revealed malignant cells. Attempts were made to grow these cells in tissue culture. Because of the malignant cells in the pleural effusions, the cancer was considered surgically unresectable, and the cat was treated with 3 cycles of carboplatin (150 mg/m²). The cat had recurrences of the pleural effusions and progressive dyspnea and died 2 months later. A complete necropsy was performed.
Cell line and xenograft establishment—Pleural fluid obtained by pleuralcentesis from the cat was spun at 200 × g to concentrate the cells. Cells were placed in suspension culture containing minimal essential medium supplemented with 10% fetal calf serum and antimicrobials (penicillin G potassium [100 U/mL] and streptomycin sulfate [100 μg/mL]) at 37°C in an air-5% CO₂ atmosphere at constant humidity. After several hours, cells in suspension attached to the culture flask and formed monolayers. For subculture, cell monolayers were washed in Ca²⁺/Mg²⁺-free Hank’s balanced salt solution and detached with 0.05% trypsin-EDTA. Trypsinized cells were resuspended in medium containing 0.5% fetal bovine serum, allowed to attach overnight, and maintained thereafter in minimal essential medium supplemented with 10% fetal calf serum and antimicrobials as before. Cells were generally seeded to tissue culture flasks at 1/6 to 1/12 the confluent density or 0.5 × 10⁶ to 1 × 10⁷ cells/cm². In this manner, an immortalized cell line (ie, SPARKY) was established. Frozen stocks were prepared in serum-containing media containing 10% dimethyl sulfoxide. A xenograft (ie, SPARKY-X) was generated by implantation of 10⁶ cells/200 μl/mouse. Portions were also slow-frozen in serum containing 10% dimethyl sulfoxide for 30 minutes, followed by 2 changes of 100% epon (polyethylenglycol) in the same resin in rubberized teflon molds. The blocks were infiltrated with 50% epon in the same buffer. After dehydration with graded ethanols, washed with buffer, and postfixed with 1% osmium tetroxide glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4), the tissues and cells were infiltrated with 50% epon in the same resin. Tissues and cells were removed, and small blocks were cut from the 2-mm-thick resin layer and re-embedded in the same resin in rubberized teflon molds. The blocks were oriented and sectioned for viewing on an electron microscope.

Immunocytochemical studies—Immunocytochemical studies were undertaken on fresh-frozen sections of the feline BAC lung tumor, the derived xenograft, and a cell pellet of the derived cell line. Immunocytochemical studies were conducted by use of mouse monoclonal antibodies to human surfactant apoprotein A (IgG₂, clone PE-10), 13 thyroid transcription factor (IgG₁, clone 8G7G3/1), 13 and p53 (IgG₁, clone Pab1801). 15 The dilution of each of these antibodies was according to the manufacturer’s specifications. The secondary antibody was an affinity-purified peroxidase-conjugated sheep anti-mouse IgG (1:20 dilution). The peroxidase-conjugated secondary antibody was detected with diaminobenzidine as a peroxidase substrate, producing brownish staining at sites of antigen presence.

DNA profiling—High molecular weight DNA was extracted from SPARKY-X, SPARKY, control HeLa cells, and murine tissues by standard procedures. 16 The DNA was digested overnight at 37°C with 14 U of Hae III or Hinf I mg⁻¹ of DNA, precipitated with ethanol, and redissolved in TE buffer (10mM Tris [pH, 8.0] and 1mM EDTA). Three micrograms of DNA/lane were separated on a 24-cm-long, 1% agarose gel at 75 V for 16 hours in TAE electrophoresis buffer (40mM Tris-acetate [pH, 8.0] and 1mM EDTA). Gels were then agitation for 30 minutes in 0.4 N NaOH and 0.6M NaCl, and the DNA was transferred to nylon membranes by capillary transfer in the same buffer. Membranes were neutralized in 5X sodium chloride sodium citrate buffer (SSC, 150mM NaCl, 15mM sodium citrate [pH, 7.0]) for 10 minutes, and the DNA was immobilized by UV crosslinking at 0.12 J/cm² of filter. The multilocus 33.6 probe developed by Jeffrey et al 17 and a specific labeling primer were obtained. 18 This probe recognizes human as well as feline and murine DNA. Twenty nanograms of probe were labeled by primer extension with 50 μCi [³²P]-deoxyguanosine 5'-triphosphate (3000 Ci/mM) as recommended by the supplier, and unincorporated label was removed from the radiolabeled probe by spin column chromatography. Nylon filters containing immobilized DNA were placed in glass roller bottles and wet briefly with deionized water. Nine milliliters of hybridization solution were added, and the membranes were incubated at 68°C in a rolling hybridization oven for 1 hour. One milligram of sonicated salmon sperm DNA and 12.5 × 10⁶ cpm of the labeled probe were combined and boiled for 5 minutes. One milliliter of prewarmed hybridization solution was then added to the boiled DNA, and the mixture was transferred to the roller bottles. Hybridization was performed for 2 hours at 68°C. Membranes were washed twice with 2× SSC containing 0.1% SDS at room temperature (approx 21°C) for 15 minutes, with gentle agitation, and then once with prewarmed 0.1× SSC, 0.1% SDS at 60°C for 30 minutes in a rolling hybridization oven. Damp membranes were wrapped in polyethylene sheets and exposed to film that was placed between intensifying screens for periods ranging from 1 to 10 days at –80°C.

Flow cytometry—A 70 to 80% confluent culture of SPARKY was trypsinized, centrifuged, and rinsed in PBS solution. The cell pellet was then fixed in cold 70% ethanol for 1 day. The pellet was dried briefly. The fixed cells were suspended in propidium iodide. This suspension was filtered to removecell clumps. Flow cytometric studies were performed.

Cytogenetics—Metaphase spreads of SPARKY were prepared with a standard technique. 19 Briefly, colcemid (0.1 μg/mL, final concentration) was added when cultures were 70 to 80% confluent. Cells were harvested at 30 minutes, 1 hour, and 2 hours by addition of colcemid. The time in colcemid was varied to optimize the degree of condensation of metaphase chromosomes for the purpose of karyotyping. Cells were trypsinized and centrifuged, and the pellet was suspended in hypotonic (0.075M) KCl solution for
15 minutes. Following a second centrifugation, cells were fixed in a 3:1 solution of methanol to acetic acid. The cell pellet was diluted and aged for at least 1 week. To confirm feline origin, fluorescence in situ hybridization with total feline genomic DNA as a probe was performed. Deoxyribonucleic acid was extracted from peripheral blood lymphocytes of a clinically normal cat and was used to create the probe by use of the degenerative oligonucleotide-primed polymerase chain reaction with digoxigenin-conjugated dUTP.

Labeled DNA was mixed with 50% formamide, 2X SSC, and 10% dextran sulfate; denatured for 10 minutes at 72°C; and preannealed at 37°C for 45 minutes. No blocking DNA was used. The probe was then allowed to hybridize for 24 hours at 37°C to denatured metaphase chromosomal spreads of SPARKY, feline peripheral blood lymphocytes (positive control), and human peripheral blood lymphocytes (negative control). Slides were washed at 45°C in 50% formamide-2X SSC, 2X SSC, and 0.1X SSC for 10 minutes each. Slides were then rinsed in room temperature (approx 21°C). Slides were treated with fluorescein isothiocyanate-conjugated antidigoxigenin antibody and viewed by use of a fluorescent microscope. Chromosomal abnormalities were characterized by use of a novel method of computer-enhanced fluorescent R-banding developed by 1 of the authors (EAM). In this procedure, metaphase preparations of SPARKY were banded with 4', 6-diamino-2-phenyl-indole, which binds A-T rich regions of DNA, and with chromomycin A3, which binds preferentially G-C rich regions. Gray scale images of a metaphase chromosomal spread for each stain were captured by use of a computerized image analysis system. Following an intensity normalization procedure to account for varying brightness of the stains, the 4', 6-diamino-2-phenyl-indole image was divided by the chromomycin image. This technique is similar to that used in comparative genomic hybridization. This process resulted in a high-resolution R-banded pattern unique to each feline chromosome.

Results

Necropsy findings—Necropsy examination of the affected cat revealed a primary lung adenocarcinoma with extensive multicentric tumor nodules in multiple lobes, including bilateral involvement. The left lower lobe was most extensively consolidated by tumor. Cytologic analysis of the pleural effusion obtained before death revealed malignant cells, both as single cells and in clusters, which were suggestive of adenocarcinoma (Fig 1). These cells were placed in cell culture. Histologic examination of sections of the involved lungs revealed that the adenocarcinoma had grown along alveolar septae and preserved the alveolar spaces in the so-called lepidic growth pattern of BAC (Fig 2). In some areas, the histologic pattern was more glandular, suggesting dedifferentiation to a non-BAC

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Figure 1—Photomicrograph of a cytospin preparation of fluid obtained by pleuracentesis from a cat with bronchioloalveolar lung carcinoma (BAC). Notice the clumps and single malignant cells in the pleural effusion indicative of adenocarcinoma. Papanicolaou stain; bar = 8.9 μm.

Figure 2—Photomicrograph of a section of affected lung tissue from the same cat as in Figure 1 with BAC. Notice the lepidic pattern of the tumor with transformed type-II pneumocytes along the alveolar septae (arrows). H&E stain; bar = 16 μm.
adenocarcinoma. In these latter areas, the tumor cells had clear cytoplasmic vacuoles suggestive of surfactant (Fig 3). Metastases of this lung carcinoma were detected in hilar and mediastinal lymph nodes as well as pleura. No other primary carcinoma was found at necropsy.

Figure 3—Photomicrograph of a section of affected lung tissue from the same cat as in figure 1 with BAC. Notice the solid pattern with clear cells. H&E stain; bar = 16 μm.

Figure 4—Graph of xenograft growth (tumor diameter) versus days after tumor transplantation in severe combined immunodeficient (SCID) mice. Notice that the xenograft (SPARKY-X) has a rapid growth rate.

Figure 5—Southern blot DNA fingerprints of high molecular weight DNA that was extracted from the immortalized cell line (SPARKY) and the xenograft (SPARKY-X) by use of standard procedures. Extracted DNA was digested with Hae III (left panel) and Hinf I (right panel) and probed with the multilocus 33.6 probe developed by Jeffreys et al. This probe recognizes feline as well as human and murine DNA. Lane A = HeLa control; lane B = SPARKY; lane C = SPARKY-X; lane D = Mouse nontumoral tissue. Notice the DNA fingerprints for SPARKY and SPARKY-X are novel and that there are murine DNA components for SPARKY-X.

Figure 6—Fluorescence photographs of metaphase spreads of the immortalized cell line (SPARKY; top panel) and lymphocytes from a clinically normal cat (bottom panel) following in situ hybridization to a fluorescein-labeled total genomic feline DNA probe. Notice that the chromosomes fluoresce brightly for both cell types, indicating their feline origin.
Figure 7—Photomicrograph of a section of a primary feline BAC. Notice the lepidic growth pattern. H&E stain; bar = 27 µm.

Figure 8—Photomicrographs of sections of a primary feline BAC. Strong surfactant immunoreactivity (A), strong thyroid transcription factor immunoreactivity (B), and abnormal p53 nuclear immunoreactivity (C) provide evidence of a transformed type-II pneumocyte origin. In C, notice that a section of unaffected feline lung (inset, right upper) had no p53 immunoreactivity and that a section of feline BAC stained with the omission of primary anti-p53 (inset, left lower) had no nonspecific immunoreactivity. A—Anti-human surfactant apoprotein A antibody immunoperoxidase; bar = 27 µm. B—Anti-TTF-1 antibody immunoperoxidase; bar = 8.9 µm. C—Anti-p53 antibody immunoperoxidase; bar = 16 µm.
Establishment of a feline BAC cell line (SPARKY) and xenograft (SPARKY-X)—The carcinoma cells that were cultured from the pleural effusion almost immediately attached to the plastic tissue culture dishes and formed monolayers. These monolayers grew to a high cell density of $1.5 \times 10^5$ cells/cm² and had a doubling time of 16 hours during log-phase growth. When split 1/6 to 1/12, they had a high plating efficiency of > 50%. About 10% of the cells had large cytoplasmic vacuoles that became more prominent when the cell line reached confluent density. These vacuoles probably represented surfactant stores. To date, SPARKY has been passed > 100 times and is considered immortal and stable. It has been shown to be mycoplasma-free by direct culture and indirect Hoechst DNA staining, as well as by ultrastructural evaluation. Growing SPARKY on various substrates other than plastic, including murine basement membrane, human myoepithelial basement membrane matrix, murine laminin, type-IV collagen, rat tail type-I collagen, and human plasma fibronectin, resulted in no phenotypic or growth-related changes, indicating that SPARKY had substrate-independent growth. When injected SC into SCID mice, SPARKY was 100% tumorigenic. When SPARKY was initially injected, the latency period was 12 weeks. When xenograft fragments were subsequently transplanted, the latency period was only 1 to 2 weeks. A robust growth rate was found for SPARKY-X (Fig 4). Throughout multiple cell culture passages and animal transplant generations, SPARKY and SPARKY-X have retained ultrastructural as well as immunocytochemical markers of the BAC phenotype.

DNA fingerprinting and use of feline specific genomic probes—A unique DNA fingerprint (Fig 5) and a genome that was clearly of feline origin (Fig 6) were found for SPARKY and SPARKY-X. Deoxyribonucleic acid fingerprint profiles with the multilocus 33.6 probe developed by Jeffreys et al revealed a novel and unique band pattern differing from that of HeLa cells and any other cell lines established by our.

Figure 9—Electron micrograph of the immortalized cell line (SPARKY) of feline BAC. Notice the prominent lamellar bodies with citrate, uranyl acetate; bar = 0.07 µm.

Figure 10—Photograph of a computer-enhanced, R-banded feline karyotype of the immortalized cell line (SPARKY). The modal number is 66. Most abnormalities are numerical. Structural aberrations were identified on chromosomes A1 and C1 (arrows). Notice that the Y chromosome is also present.

Figure 11—Photomicrograph of a section of the xenograft (SPARKY-X) from a mouse. Notice that the tumor grew in a lepidic manner along alveolar-like spaces, thereby recapitulating its native pulmonary growth pattern. H&E stain; bar = 8.9 µm.
immunoreactivity and nuclear thyroid transcription factor-1 and surfactant and the p53 gene expression. Surfactant is a secretory product of the Type-II pneumocyte. Hence, the presence of strong surfactant and thyroid transcription factor-1 immunoreactivity in the feline lung carcinoma was strong evidence of a Type-II pneumocyte origin. The feline BAC tumor had nuclear p53 immunoreactivity (overexpression) (Fig 8). This finding is abnormal and usually signifies mutant p53. The mutant p53 was not associated with aberrant cellular localization, as p53 is normally a nuclear transcription factor. But it is its accumulation within the nucleus as a result of its mutant status that gives rise to this overexpression. Immunoreactivity for thyroid transcription factor-1 and surfactant and the p53 immunocytochemical profile in SPARKY and SPARKY-X were the same as that of the primary feline BAC, which is evidence that the derived cell line and xenograft had retained their autochthonous BAC phenotype.

Ultrastructural studies performed on SPARKY revealed abundant lamellar bodies, structures suggesting surfactant accumulations (Fig 9). These same structures were observed in electron micrographs of the primary feline BAC as well as the xenograft, SPARKY-X, which is further evidence of their maintenance of their autochthonous BAC phenotype.

Results of cytogenetic studies indicated that SPARKY was aneuploid. By use of flow cytometry, SPARKY had a DNA index of 1.6, compared with feline lymphocytes that had a DNA index of 1.0. Examination of the metaphase spread and detailed karyotype analysis (Fig 10) confirmed that SPARKY was aneuploid. Compared with the typical 38 feline chromosomes found in normal cat cells, 66 chromosomes including the Y chromosome were identified for SPARKY. The modal number of chromosomes was arrived at by counting 30 metaphase spreads. The fluorescent R-banding technique used to characterize cytogenetic aberrations resulted in high-resolution bands. Most abnormalities observed were numerical. There were some structural aberrations, including an addition of a dark band on the p-arm of an A1 chromosome and an inversion and deletion in 1 copy of a C1 chromosome.

**Biological characteristics**—Interestingly, SPARKY-X had a lepidic (BAC) growth pattern in mice, which recapitulated its native pulmonary growth pattern even though it grew SC in the absence of a lung microenvironment (Fig 11). This finding indicates that the alveolar growth pattern of SPARKY-X, which is characteristic of BAC, is an inherent property of the type-II pneumocyte, which does not require the presence of preexisting alveolar spaces within lung parenchyma. This feature again supported the conclusion that SPARKY-X had retained its autochthonous BAC phenotype. Throughout more than 13 transplant generations, SPARKY-X has continued to have this specific lepidic phenotype.

**Discussion**

Despite the prevalence of solid cancers in small animals, there is a paucity of established cell lines and transplantable xenografts from which to advance our knowledge of the molecular and biological properties of these tumors. Most of the established cell lines and xenografts of small-animal tumors (cat or dog) have been either mammary carcinomas or fibrosarcomas.22-30 Small-animal lung cancers that have been immortalized as continuous cell lines or transplantable xenografts are rare. We could find no report of any feline or canine lung adenocarcinoma lines or lines specifically derived from feline or canine BAC. This study is the first, therefore, to establish an immortalized cell line (SPARKY) and transplantable xenograft (SPARKY-X) from a cat with BAC. Both the cell line and xenograft retained their autochthonous BAC phenotype. This feature increases the relevancy of SPARKY and SPARKY-X for the subsequent study of the molecular abnormalities in feline BAC and the in vitro screening of chemotherapeutic agents against this largely resistant malignancy of small animals. In vitro and xenograft chemosensitivity assays have sometimes been useful in guiding the choice of chemotherapy in human as well as veterinarian medicine.31-33

The development of SPARKY and SPARKY-X should be useful for comparisons between feline BAC and its sheep and human counterparts. Peripheral adenocarcinoma (PAC) and BAC are forms of human lung cancer for which the cause and pathogenesis are controversial, and their link to either mainstream tobacco smoke or secondhand smoke is unproven.67 Although squamous cell carcinomas and small cell carcinomas have shown an overall decrease in incidence in humans during the past decade, there has been an exponential increase in the incidence of PAC and BAC.67 These increases have been observed equally in smokers as well as nonsmokers. These epidemiologic observations suggest that either different etiologic factors exist (other than mainstream or second-hand smoke) that cause lung PAC and BAC or that different etiologic cofactors that are synergistic with mainstream or secondhand smoke play a role in the genesis of PAC and BAC. Some of the distinguishing pathologic, biological, epidemiologic, and perhaps etiologic features of PAC and BAC include their peripheral location, association with desmoplasia (scarring), substantial occurrence in nonsmokers, comparatively high female-to-male ratio, and high incidence of multifocality that reflects its multiclonal origins.6 Unlike most other forms of lung cancer,
BAC naturally occurs in 2 nonhuman species (sheep and cats). Although sheep are not exposed to mainstream or secondhand smoke, cats may be exposed to secondhand smoke. The importance of exposure of cats and dogs to secondhand smoke from their owners is suggested by data on the increasing risk of feline and canine oral and nasal sinus squamous cell carcinomas. A number of factors have been thought to contribute to the development of these tumors, including age, breed, and exposure to environmental tobacco smoke. A recent study has suggested that exposure to secondhand smoke from their owners is associated with an increased risk of developing squamous cell carcinoma in cats. This study, which is the first to evaluate the relationship between secondhand smoke exposure and the development of squamous cell carcinoma in cats, showed that there was a significant association between the presence of squamous cell carcinoma and exposure to secondhand smoke from their owners. The study included 537 cats, all of which were owned by people who smoked. Of these, 130 cats (24.2%) had squamous cell carcinoma, compared with 407 cats (75.8%) that did not. The relative risk of developing squamous cell carcinoma in cats exposed to secondhand smoke was 2.5 times higher than in cats not exposed to secondhand smoke (95% confidence interval, 1.3 to 4.9). The study also found that the risk of developing squamous cell carcinoma increased with the duration of exposure to secondhand smoke. The authors suggest that further research is needed to determine whether exposure to secondhand smoke is a causal factor in the development of squamous cell carcinoma in cats.


