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α -Smooth muscle actin expression in cancer-associated fibroblasts in canine epithelial tumors

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Abstract

Tumor tissues contain not only cancer cells but also other cell types including, fibroblasts, immune cells, and endothelial cells, which interact with cancer cells. In human medicine, cancer-associated fibroblasts (CAFs) have been reported to promote tumor growth. CAFs are known to express α -smooth muscle actin (α -SMA), and this expression is correlated with poor prognosis in humans with cancer. However, the role of CAFs in canines and α -SMA expression in canine CAFs remains unknown. This study evaluated whether CAFs are present within the stroma of various types of canine epithelial tumors, for example, mammary gland tumors, squamous cell carcinoma, and anal sac adenocarcinoma, and assessed α -SMA expression in CAFs isolated from canine epithelial tumors. α -SMA analysis of tumor tissues revealed a cytoplasmic localization with variable levels of expression. α -SMA was detected in 60.9% (14/23) of epithelial tumor tissues and in 80% (8/10) of anal sac adenocarcinoma tissues. CAFs and normal fibroblasts (NFs) were isolated from tumor and skin tissues. The size of CAFs was variable, and most CAFs had large cell volume, in contrast to NFs. Most CAFs expressed α -SMA stress fibers and had higher α -SMA protein levels than NFs. Taken together, our findings provide evidence that canine CAFs express α -SMA in various canine epithelial tumors. Further studies are required to investigate the correlation between canine CAFs and clinical parameters and to elucidate the mechanisms underlying the effects of CAFs on cancer progression.

Key Words: α -smooth muscle actin, cancer-associated fibroblasts, tumor microenvironment

Introduction

Tumor tissues contain not only cancer cells but also other cellular and noncellular components^{9,26)}. The cellular components of the tumor microenvironment include fibroblasts, immune cells, and endothelial cells^{9,26)}. The tumor

stroma cells that surround the cancer cells create the tumor microenvironment, which contributes to the malignant features of cancer cells^{10,21,26)}.

Cancer-associated fibroblasts (CAFs) comprise a large proportion of the cellular components of the tumor microenvironment²⁶⁾. Recent studies have revealed that CAFs contribute to tumor

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proliferation, invasion, and metastasis via secretion of various growth factors, cytokines, and chemokines^{2,11,23}. CAFs are activated fibroblasts that share similarities with fibroblasts stimulated by inflammatory conditions or activated during wound healing³. The most widely used marker for CAFs is α -smooth muscle actin (α -SMA), which is also a specific marker for myofibroblasts²³. Studies have indicated a significant correlation between a CAF-rich stroma and adverse survival prognostic factors in many human cancers, such as breast cancer and squamous cell carcinoma (SCC)^{3,15}.

CAFs play important roles in tumor growth. They are genetically stable and develop less drug resistance than cancer cells^{5,20}. Therefore, CAFs may serve as an effective therapeutic target in cancer^{5,20}. In human medicine, several studies have corroborated the therapeutic potential of targeting the tumor progression-supporting functions of CAFs^{7,26}. Several agents such as Nintedanib and AMD070, that target the soluble mediators of interactions between CAFs and cancer cells, have been tested in preclinical studies or clinical trials^{5,7,26}.

In veterinary medicine, only a few studies have been performed on CAFs. In one study, α -SMA immunostaining helped identify α -SMA-positive CAFs in 74.5% (35/47) of the feline oral SCCs¹². Moreover, cats with α -SMA-positive CAFs (35 days) had a modest, but significantly shorter, median survival time than cats with α -SMA-negative CAFs (48.5 days)¹². Another study showed that co-culturing mammary gland tumor (MGT) cells and the CAFs isolated from MGT tissues led to significant changes in the expression of cancer-related genes associated with proliferation, metastasis, and angiogenesis¹³. However, whether α -SMA-positive CAFs are present in canine epithelial tumors is not known.

Therefore, the current study evaluated whether CAFs are present in the stroma of various canine epithelial tumors and assessed α -SMA expression in CAFs isolated from canine epithelial tumors.

Materials and methods

Archival tumor tissue collection: Formalin-fixed paraffin embedded canine epithelial tumor tissues, which were surgically resected at the Hokkaido University Teaching Hospital between October 2014 and October 2016, were collected. All tissues were examined with hematoxylin and eosin staining to confirm the diagnosis. These tissues were used for immunohistochemistry (IHC).

IHC: Tumor sections of the freshly cut (3- to 5- μ m-thick sections) formalin-fixed paraffin embedded tissue were mounted on slides, deparaffinized, and activated by microwaving for 10 min. Endogenous peroxidase activity was blocked by a 15-min incubation in 0.3% H₂O₂. The sections were reacted with 10% rabbit serum (Nichirei Bioscience, Tokyo, Japan) for 1 hr. Immunohistochemistry was then performed using an anti- α -SMA mouse monoclonal antibody (clone 1A4) (catalog number A2547, Sigma-Aldrich, St-Louis, MO, USA) at a dilution of 1 : 2,000. The sections were incubated with antibodies overnight at 4°C, following which they were incubated with biotinylated rabbit anti-mouse antibody (catalog number 424022, Nichirei Bioscience) for 1 hr at room temperature (15–25°C) and then with streptavidin-biotinylated peroxidase complex (Nichirei Bioscience) for 5 min at room temperature. The chromogen diaminobenzidine was used for visualization for 90 sec at room temperature. The sections were then counterstained with hematoxylin. α -SMA expression in each section was observed under an optical microscope.

Fresh tumor tissue collection for cell culture: Tumor and skin tissues were obtained from dogs diagnosed with epithelial tumors for which surgical resection had been performed at the Hokkaido University Veterinary Teaching Hospital between May 2016 and October 2016; written consent had been obtained from the owners before inclusion in the study. CAFs and NFs were isolated from the tumor and skin tissues from

these animals. All tissues were pathologically examined with hematoxylin and eosin staining to confirm the diagnosis.

Isolation and identification of CAFs and NFs:

Fibroblasts were isolated from epithelial tumor tissues and skin tissues in reference to previous reports^{16,29}. Canine epithelial tumor tissues and skin tissues were minced into small pieces and digested in 1 mg/ml collagenase IV (Sigma-Aldrich) with 0.5% bovine serum albumin (BSA) at 37°C for 2 hr. Undigested tissue was removed by filtration through a 100 µm-mesh, and the cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Wako, Osaka, Japan) at 37°C with 5% CO₂. Fibroblasts were separated from epithelial cells on the basis of differences in the time required for detachment by 1 mM EDTA-4Na and 0.25% trypsin. Fibroblasts often detached earlier than epithelial cells. If a high-purity fibroblast suspension could not be obtained at any point, the remaining cells were cultured and then cell separation was attempted again. The isolated cells were used for identifying fibroblasts on the basis of cell morphology. Fibroblasts were used for each experiment within four passages, and there was no more than one passage difference between CAFs and NFs.

Immunofluorescence (IF): Cells were seeded at 1.0×10^4 – 1.0×10^5 on poly-L-lysine-coated coverslips (Matsunami Glass, Osaka, Japan) in 24-well plates, cultured for 48–72 hr, and fixed with paraformaldehyde at room temperature for 1 hr. The cells were subsequently permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS), blocked with 1% BSA, and immunostained overnight at 4°C with primary mouse antibodies for vimentin (a marker for mesenchymal cells) (catalog number N1521, Dako, Tokyo, Japan), cytokeratin (a marker for epithelial cells) (catalog number N1590, Dako), and α -SMA (Sigma-Aldrich) diluted 1 : 2,000 in PBS. The cells were

incubated at room temperature for 1 hr with fluorochrome-conjugated secondary goat anti-mouse antibody diluted 1 : 500 (Alexa-Fluor-488, catalog number A21202, Thermo Fisher Scientific, Waltham, MA, USA), and the nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Dojindo, Kumamoto, Japan) at room temperature for 15 min. Coverslips were mounted on the glass slides. Images were obtained with a confocal microscope (LSM700, Zeiss, Oberkochen, Germany).

Total protein extraction and western blotting: Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Wako) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and mixed with 4× sample buffer (Wako). The samples were run on sodium dodecyl sulfate-polyacrylamide electrophoresis gels containing 12% acrylamide and blotted onto a polyvinylidene membrane (Bio-Rad, Hercules, CA, USA). Then, the membranes were blocked with 3% skimmed milk and probed at room temperature for 2 hr with the primary mouse anti- α -SMA antibody diluted 1 : 10,000 or with an anti- β -actin antibody (catalog number MAB1501, Merck Millipore, Hessen, Germany) diluted 1 : 1,000, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1 : 10,000 (General Electric Company, Fairfield, CT, USA). Luminescence reagents were used for visualization of protein bands (EzWest Lumi; Atto, Tokyo, Japan).

Results

IHC

Twenty-three archival tumor tissues were available for analysis. Table 1 lists the characteristics of the dogs and the pathological findings. α -SMA staining revealed cytoplasmic localization with varying levels of intensity in individual animals (Fig. 1). Some stromal fibroblasts expressed α -SMA (Fig. 1A, B), whereas others did

Table 1. Characteristics of the dogs and pathological findings

| No | Breed ^{*a} | Age (years) | Sex ^{*b} | Diagnosis ^{*c} | IHC ^{*d} |
|----|---------------------|-------------|-------------------|---------------------------|-------------------|
| 1 | MD | 11 | MC | ASAC | + |
| 2 | BG | 12 | M | ASAC | + |
| 3 | MD | 11 | FS | ASAC | + |
| 4 | MIX | 7 | MC | ASAC | + |
| 5 | MD | 13 | FS | ASAC | – |
| 6 | BG | 9 | MC | ASAC | + |
| 7 | MD | 10 | M | ASAC | – |
| 8 | TP | 11 | MC | ASAC | + |
| 9 | CKCS | 11 | MC | ASAC | + |
| 10 | YK | 10 | F | ASAC | + |
| 11 | PAP | 13 | F | TCC | – |
| 12 | ST | 9 | FS | TCC | + |
| 13 | FB | 14 | FS | TCC | + |
| 14 | MAL | 15 | FS | MGT | + |
| 15 | BC | 16 | F | MGT | + |
| 16 | BG | 13 | M | Nasal adenocarcinoma | – |
| 17 | MIX | 12 | FS | Nasal adenocarcinoma | – |
| 18 | LL | 12 | FS | Lung adenocarcinoma | – |
| 19 | MS | 10 | FS | Lung adenocarcinoma | – |
| 20 | PUG | 10 | M | Intestinal adenocarcinoma | – |
| 21 | WC | 9 | FS | Intestinal adenocarcinoma | – |
| 22 | MD | 9 | MC | Prostate adenocarcinoma | + |
| 23 | CH | 12 | MC | SCC | + |

^aMD: Miniature Dachshund, BG: Beagle, MIX: mixed breed, TP: Toy Poodle, CKCS: Cavalier King Charles Spaniel, YK: Yorkshire Terrier, PAP: Papillon, ST: Shih Tzu, FB: French Bulldog, MAL: Maltese, BC: Border Collie, LL: Labrador Retriever, MS: Miniature Schnauzer, WC: Welsh Corgi, CH: Chihuahua.

^bMC: Male castrated, M: Male, FS: Female spayed, F: Female.

^cASAC: Anal sac adenocarcinoma, TCC: Transitional cell carcinoma, MGT: Mammary gland tumor, SCC: Squamous cell carcinoma.

^dIHC: Immunohistochemistry, +: Positive, –: Negative.

not (Fig. 1C, D). α -SMA expression was detected in eight anal sac adenocarcinomas (ASAC), two transitional cell carcinomas (TCC), two MGTs, one prostate adenocarcinoma, and one SCC samples (Table 1). Overall, α -SMA expression was detected in 60.9% (14/23) of the tissue samples.

Isolation and characterization of CAFs and NFs

CAFs and NFs were successfully isolated on the basis of differences in the time required for detachment of fibroblasts and tumor cells from six dogs (Table. 2). Both types of fibroblasts displayed elongated spindle-shaped features (Fig. 2A, B).

The size of CAFs was variable, and most CAFs had a large cell volume (Fig. 2A), in contrast to NFs (Fig. 2B). If sufficient cells were obtained, the isolated fibroblasts were also characterized using IF. Both types of fibroblasts showed positive staining for vimentin (Fig. 2C, D) but negative results for cytokeratin (Fig. 2E, F), which indicated that the isolated cells were not epithelial cells but mesenchymal cells.

α -SMA expression in CAFs isolated from tumor tissue

IF staining revealed that CAFs expressed

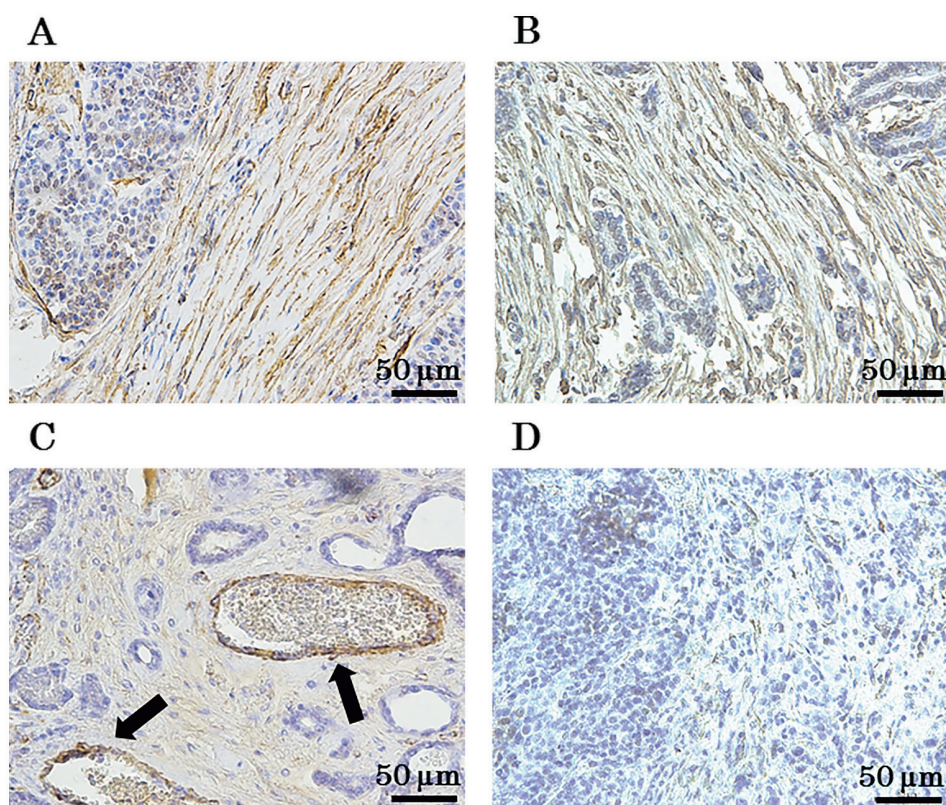


Fig. 1. Representative images of α -SMA expression in the tumor stroma. Tumor stroma fibroblasts positive for α -SMA expression (A, B). Tumor stroma fibroblasts negative for α -SMA expression (C, D). α -SMA expression in vascular smooth muscle was demonstrated as a positive control (arrows in C). A: dog 1, B: dog 14, C: dog 7, D: dog 17.

Table 2. Characteristics of the dogs from which fibroblasts were isolated

| No | Breed ^a | Age (years) | Sex ^b | Diagnosis ^c | IF ^{*d} | WB ^{*e} |
|----|--------------------|-------------|------------------|---------------------------|------------------------|------------------|
| 1 | MD | 11 | MC | ASAC | α -SMA, VIM, CK | α -SMA |
| 3 | MD | 11 | FS | ASAC | — | α -SMA |
| 20 | PUG | 10 | M | Intestinal adenocarcinoma | α -SMA | — |
| 24 | MD | 13 | F | MGT | α -SMA, VIM, CK | α -SMA |
| 25 | CH | 11 | MC | HCC | — | α -SMA |
| 26 | YK | 6 | M | SCC | — | α -SMA |

^aMD: Miniature Dachshund, CH: Chihuahua, YK: Yorkshire.

^bMC: Male castrated, M: Male, FS: Female spayed, F: Female.

^cASAC: Anal sac adenocarcinoma, MGT: Mammary gland tumor, HCC: Hepatocellular carcinoma, SCC: Squamous cell carcinoma.

^dIF: Immunofluorescence. —: not performed.

^eWB: Western blotting, —: not performed.

α -SMA stress fibers (Fig. 3A, C, E), whereas NFs were weakly stained for α -SMA (Fig. 3B, D, F). CAFs showed strong staining for α -SMA in ASAC and intestine adenocarcinoma patients. NFs from intestine adenocarcinoma weakly expressed α -SMA stress fibers. Additionally, western blotting

revealed that CAFs had higher α -SMA protein levels than NFs in four dogs (Fig. 4A–D). There was no difference in α -SMA protein levels between CAFs and NFs from the patient with SCC (Fig. 4E).

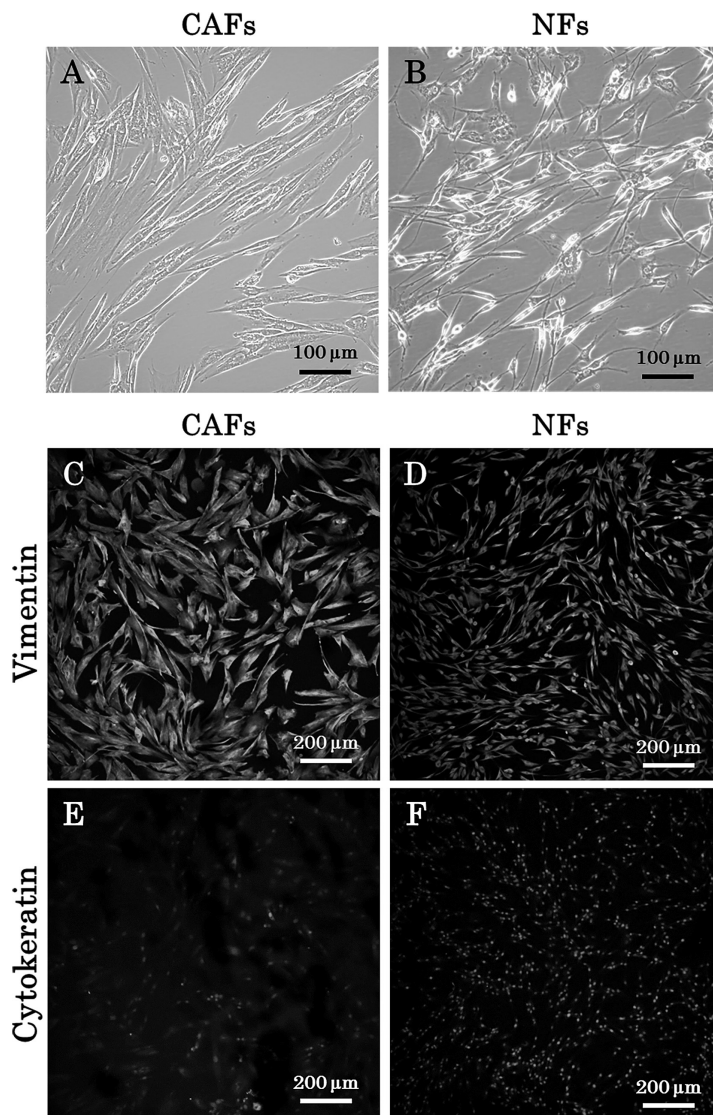


Fig. 2. Characteristics of isolated fibroblasts. The cells displayed elongated spindle-shaped features (A, B) (light microscope, $\times 200$). The CAFs size was variable and most CAFs had a large cell volume (A), in contrast to that of NFs (B). Both cell types showed staining for vimentin (C, D) but not for cytokeratin (E, F) (confocal microscope, $\times 100$).

Discussion

The current study targeted epithelial tumors since CAFs can be easily distinguished from these tumor cells because of their spindle morphology. In humans, epithelial tumors such as MGT and SCC have been mainly targeted for CAF research^{8,14,17,22}. In the current study, various canine epithelial tumors such as MGT, SCC, and ASAC were targeted to evaluate α -SMA expression in CAFs.

α -SMA expression was detected in 60.9% (14/23) of the tumor tissue samples, such as those of ASAC, TCC, MGT, prostate adenocarcinoma, and SCC; α -SMA was expressed in 80.0% (8/10) of the ASAC tissues. α -SMA-positive CAFs were often present in ASAC tissues, and they may have contributed to the malignant nature of the ASAC. Recent studies have reported that α -SMA expression was detected by IHC in 44.4–79.5% of tumor tissue samples from human patients with MGT or SCC^{1,19,28}. In humans, the most widely

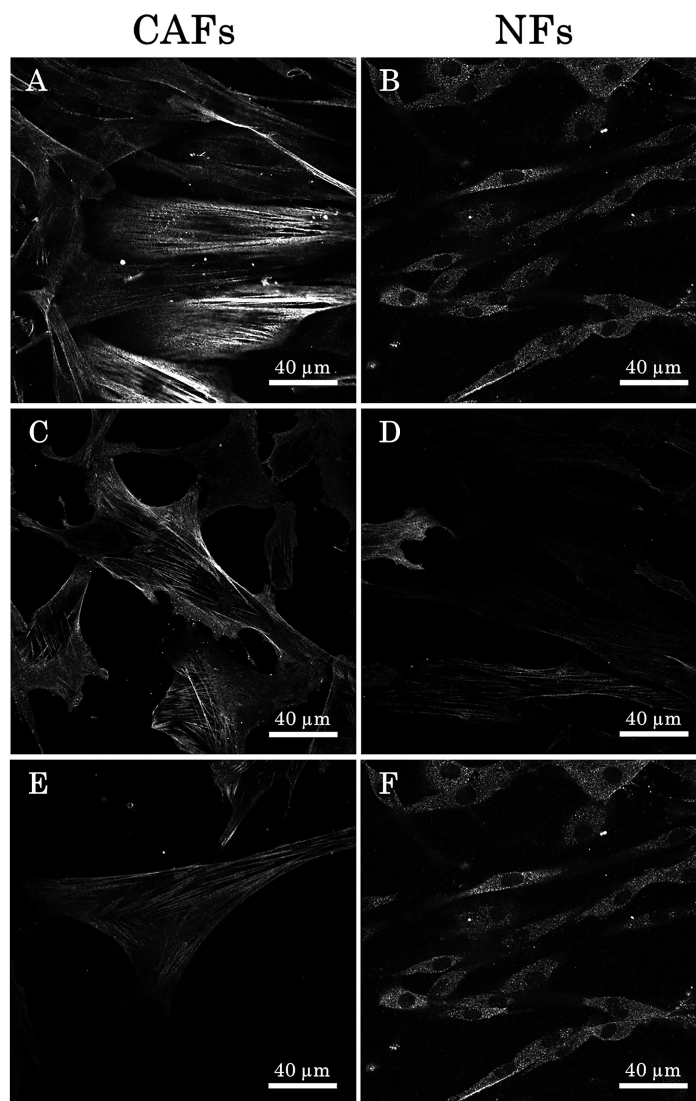


Fig. 3. α -SMA expression in fibroblasts derived from different tumors. Fibroblasts from an ASAC patient (dog 1) (A, B). CAFs expressed α -SMA stress fibers (A), and showed strong staining for α -SMA, whereas NFs were focally and weakly stained for α -SMA (B). Fibroblasts from an intestinal adenocarcinoma patient (dog 20) (C, D). CAFs expressed α -SMA stress fibers, and showed strong staining for α -SMA (C), whereas NFs expressed weakly α -SMA stress fibers (D). Fibroblasts from a MGT patient (dog 24) (E, F). CAFs expressed α -SMA stress fiber, but not strong staining for α -SMA, whereas NFs were focally and weakly stained for α -SMA (F) (confocal microscope, $\times 630$).

used marker for CAFs is α -SMA, and studies have indicated that increased α -SMA expression detected by IHC in the tumor stroma is associated with higher histologic grade, lymph node metastasis, increased micro-vessel density, and poor prognosis^{3,4,27}. However, no marker has been identified in canine CAFs. In the current study, α -SMA expression was detected in CAFs isolated from various canine epithelial tumors. Therefore, α -SMA is a possible CAFs marker in

canine tumors such as ASAC, which show high α -SMA expression, although it should be pointed out that α -SMA is not a specific marker, as it is also expressed in the vascular smooth muscle. Further studies are required to elucidate the correlation between the expression of α -SMA and other markers in canine CAFs and clinical parameters.

In the current study, CAFs were successfully isolated from canine tissues on the basis of

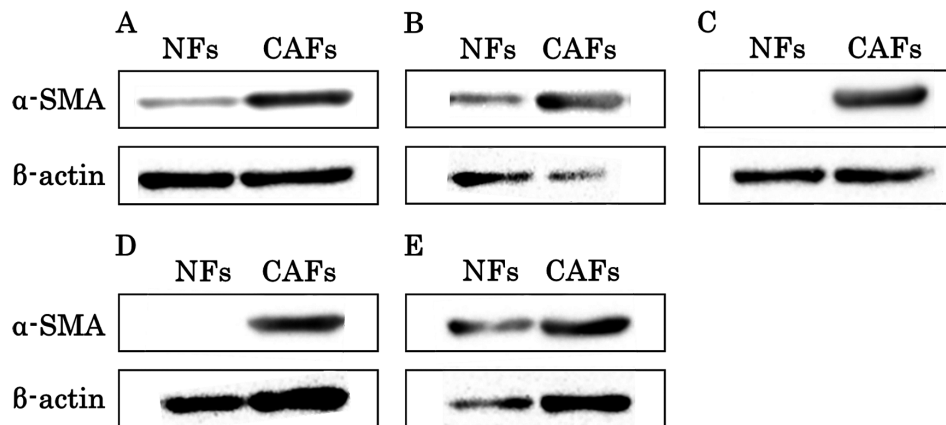


Fig. 4. Protein levels of α -SMA in fibroblasts derived from different tumors. CAFs showed higher α -SMA protein levels than NFs in four patients (A-D). There was no difference in α -SMA protein levels between CAFs and NFs from the SCC patient (E). A: dog 1, B: dog 3, C: dog 24, D: dog 25, E: dog 26.

differences in the time required for detachment of different cell types^{16,29}). The size of CAFs was variable, and most CAFs had a large cell volume, in contrast to NFs. Most CAFs exhibited higher α -SMA protein levels than NFs. These findings indicate that the characteristics of canine CAFs differed from those of canine NFs. In human medicine, CAFs have been reported to promote tumor proliferation, invasion, and metastasis *in vivo* and *in vitro*^{3,6,18}). CAFs act as a source for growth factors and cytokines known to play critical roles in cancer progression³). Cancer cells have also been known to induce the conversion of resident stromal fibroblasts and bone marrow-derived mesenchymal stem cells into CAFs^{23,24}). Moreover, through the so-called “education” by cancer cells, CAFs acquire the properties of myofibroblasts, including α -SMA expression and strong cell contractility^{25,26}). In the current study, CAFs isolated from canine tissues were positive for α -SMA, and the findings for α -SMA expression suggested that CAFs possess the properties of myofibroblasts and play critical roles in cancer progression. Further studies are required to determine what factors are secreted from canine CAFs and how these factors support the malignant progression of tumors. The limitations of this study were that the number of dogs with some types of tumors was small and that CAFs were isolated from only a few types of tumor

tissues.

Taken together, our findings provide evidence that (a) canine CAFs are present in various canine epithelial tumors and express α -SMA in the stroma, (b) CAFs had higher α -SMA protein levels than NFs, and (c) these CAFs may play critical roles in cancer progression. Further studies are required to investigate the correlation between canine CAFs and clinical parameters and to elucidate the mechanisms underlying the effects of CAFs on cancer progression. Elucidation of these factors is likely to provide veterinary practitioners additional prognostic information and lead to new anticancer treatments targeting CAFs and the cancer-stroma interactions in canine epithelial tumors.

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