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Case Report

A Case of Canine Cutaneous Clear Cell Adnexal Carcinoma with Prominent Expression of Smooth Muscle Actin

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Abstract: Cutaneous clear cell adnexal carcinoma was found in the right lip of a 14-year-old male castrated Shih Tzu. Histologically, the tumor mostly consisted of neoplastic cells with clear or vacuolated cytoplasms and contained frequent tubular structures. Neoplastic cells showed coexpression of pan-cytokeratin (CK) and vimentin by double-labeled immunofluorescence staining. In addition, immunohistochemistry revealed that the tumor cells were positive for pan-CK (AE1/AE3, KL1, CAM 5.2), CK-7, CK-8, CK-14, CK-15, CK-18, vimentin and alpha-smooth muscle actin (SMA) with varied intensity and positivity. Among these marker proteins, SMA was positive in 75% of the tumor cells. On the other hand, CK-15, which is a specific marker of follicular stem cells, was expressed in less than 1% of the tumor cells. Based on these findings, the tumor showed diverse differentiation in apocrine sweat glands and the inner and outer root sheaths of hair follicles, indicating the follicular stem cell to be the origin of this tumor. (J Toxicol Pathol 2010; **23**: 265–269)

Key words: dermatopathology, dog, cytokeratin, skin, skin tumor, stem cell

Introduction

Canine cutaneous tumors that consist of clear or vacuolated cells are uncommon and include balloon cell melanoma, clear cell basal carcinoma, sebaceous carcinoma, apocrine sweat gland adenoma clear cell variant, clear cell trichoblastoma and canine cutaneous clear cell adnexal carcinoma[1–5]. Canine cutaneous clear cell adnexal carcinoma has been described as clear cell hidradenocarcinoma[5] and follicular stem cell carcinoma[4], as first proposed by Schulman *et al*. in 2005[5]. This neoplasm does not differentiate into a single, special cutaneous adnexa, but has multilineage potential that enables it to differentiate into multiple cutaneous adnexa[4,5]. Immunohistochemical examination shows that neoplastic cells coexpress cytokeratin (CK) and vimentin, indicating that the neoplasm may be derived from follicular stem cells[4–6]. Follicular stem cells in dogs differentiate into cutaneous adnexa, such as the inner and outer root sheaths, sebaceous glands and apocrine sweat glands with different CK isoform expression in the respective cutaneous adnexa[6–8]. These differences in CK expression are useful for examining divergent adnexal differentiation of tumor[3].

In this paper, we report the histological and immunohistochemical findings of a tumor in the lower lip of 14-year-old male castrated Shih Tzu that was diagnosed with cutaneous clear cell adnexal carcinoma displaying several characteristic immunohistochemical features. We show divergent adnexal differentiation of the neoplastic cells and compare the morphology with previously reported canine cutaneous clear cell adnexal carcinomas.

The tissue specimen was fixed in 10% neutral formalin and embedded in paraffin or Optimal Cutting Temperature (O.C.T.) compound (Sakura Finetek, Tokyo, Japan). A block embedded in O.C.T. compound was snap frozen and kept at –80°C. Paraffin sections were stained with hematoxylin and eosin and reacted with periodic acid Schiff (PAS). Frozen sections were stained with Sudan III. Paraffin sections were also used for immunohistochemistry. Table 1 shows the primary antibodies used in this study. As secondary antibodies, we used peroxidase-conjugated anti-mouse (Histofine Simple Stain MAX-PO(M), Nichirei, Tokyo, Japan) and peroxidase-conjugated anti-rabbit (Histofine Simple Stain MAX-PO(R), Nichirei) immunoglobulin (Ig)G. Immunoreactions were visualized by diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin. To examine coexpression of CK and vimentin within the neoplastic cells, anti-CK (clone AE1/AE3) and anti-vimentin antibodies were used for double-labeled immunofluorescence. Anti-CK and

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Table 1. Antibodies Used in This Study

Antibody	Clone	Dilution	Antigen retrieval [a]	Source [b]
Cytokeratin	AE1/AE3	1:50	Trypsin	Dako
Cytokeratin	KL1	1:100	MW	Immunotech
Cytokeratin	CAM 5.2	Prediluted	Proteinase K	Becton Dickinson
Cytokeratin 7	OC-TL 12/30	Prediluted	Proteinase K	Dako
Cytokeratin 8	Ks8.7	Prediluted	MW	Progen Biotechnik
Cytokeratin 14	LL002	1:100	MW	Serotec
Cytokeratin 15	CBL272	1:100	NT	Millipore
Cytokeratin 16	LL025	1:50	MW	Thermo
Cytokeratin 18	Ks 18.4	Prediluted	Proteinase K	Progen Biotechnik
Cytokeratin 20	Ks 20.8	Prediluted	Proteinase K	Dako
Vimentin	V9	1:25	MW	Dako
α-SMA	1A4	1:50	NT	Dako
S-100a	polyclonal	1:200	MW	Dako
NSE	NSE-1G4	1:100	MW	Zymed
PGP 9.5	13C4	1:100	MW	Ultra Clone Limited
Melan A	A103	1:50	MW	Dako
MHC classII	TAL.1B5	1:100	MW	Dako

[a] MW=microwave/citrate buffer (pH 6.0); NT=no treatment; [b] Dako, Copenhagen, Denmark; Immunotech, Marseille, France; Becton Dickinson, Heidelberg, Germany; Progen Biotechnik, Heidelberg, Germany; Serotec, Wiesbaden, Germany; Thermo Scientific, Fremont, CA, USA; Zymed, Laboratories, San Francisco, CA, USA.

anti-vimentin antibodies were labeled with affinity-purified goat anti-mouse IgG fluorescein isothiocyanate (EY Laboratories, San Mateo, CA, USA) and affinity-purified goat anti-mouse IgG (Rhodamine conjugate, Chemicon, Temecula, CA, USA), respectively.

The excised mass was 8×8×10 mm in size, and its cut surface contained multiple lobules and was white (Fig. 1). Histologically, the neoplasm was located in the dermis and was composed of lobular structures separated by thin fibrous stromal tissues. The tumor mainly consisted of round to polygonal neoplastic cells. The cells varied in size and were characterized by clear or vacuolated cytoplasms with round to oval nuclei (Fig. 2a). The cytoplasm of the cells contained PAS-positive granules (Fig. 2c), which disappeared with diastase treatment. All neoplastic cells were negative for Sudan III. There were no follicular papillary mesenchymal bodies, but there were many tubular structures with or without PAS-positive basement membrane-like structures within the neoplasm (Fig. 2b). The mitotic rate was 1–2 mitoses per 10 high-power fields.

Table 2 shows the results for the special stains and immunohistochemical staining of the tumor and normal skin tissues. Immunohistochemically, the neoplastic cells were positive for pan-CK (AE1/AE3, KL1, CAM 5.2), CK-7, CK-8, CK-14, CK-15, CK-18, vimentin and α-SMA (Figs. 3, 4). The proportion of α-SMA-positive cells was more than 75% of the neoplastic cells. However, α-SMA-positive cells were rarely observed in a few neoplastic lobules (Fig. 4).

Tubular structures in the neoplasm were positive for pan-CK (AE1/AE3, CAM 5.2, KL1), CK-7, CK-8, CK-14, CK-18, and partly positive for vimentin, but negative for α-SMA (Figs. 3, 4). A few neoplastic cells were positive for S-100 and PGP9.5, but negative for NSE and Melan A. MHC class II-positive cells were present in the neoplasm, which might represent dermal dendritic cells. Double-labeled immunofluorescence staining revealed that most neoplastic cells were double-positive for CK and vimentin (Fig. 5).

Histologically, neoplastic tubular structures were frequently observed, suggesting that neoplastic cells differentiated into apocrine sweat glands. However, the immunohistochemical results showed that the neoplastic cells expressed specific CK isoforms of several cutaneous adnexa. The tumor cells expressed CK-8 and CK-18, and had glycogenetic granules in the cytoplasm. In normal canine skin, the outer root sheath and apocrine sweat glands stain positive for CK-8. Specifically, CK-18 is expressed in apocrine sweat glands[6–8]. Glycogenetic granules are known to exist in the outer root sheath of normal canine skin. This suggests that the present tumor had differentiated into the outer root sheath and into the apocrine sweat gland. In addition, the neoplastic cells expressed pan-CK (KL1) and CK-14. In normal canine skin, pan-CK (KL1) is expressed in the superficial layer of the epidermis and inner root sheath, and CK-14 is expressed in the basal layer of the epidermis, outer root sheath, inner root sheath, sebaceous gland and myoepithelial cells of the apocrine sweat gland[6, 7]. Meanwhile, the present neoplasm was negative for Sudan III. Therefore, this tumor might not have differentiated into sebaceous glands. Immunostaining indicated that the present tumor differentiated into the epidermis, inner root sheath, outer root sheath and apocrine sweat gland and that it also coexpressed CK and vimentin. This supports the hypothesis that clear cell adnexal carcinoma derives from the follicular stem cell[6].

In previously reported cases of clear cell adnexal carcinomas, tumor cells have been negative for α-SMA[4–6]. How-

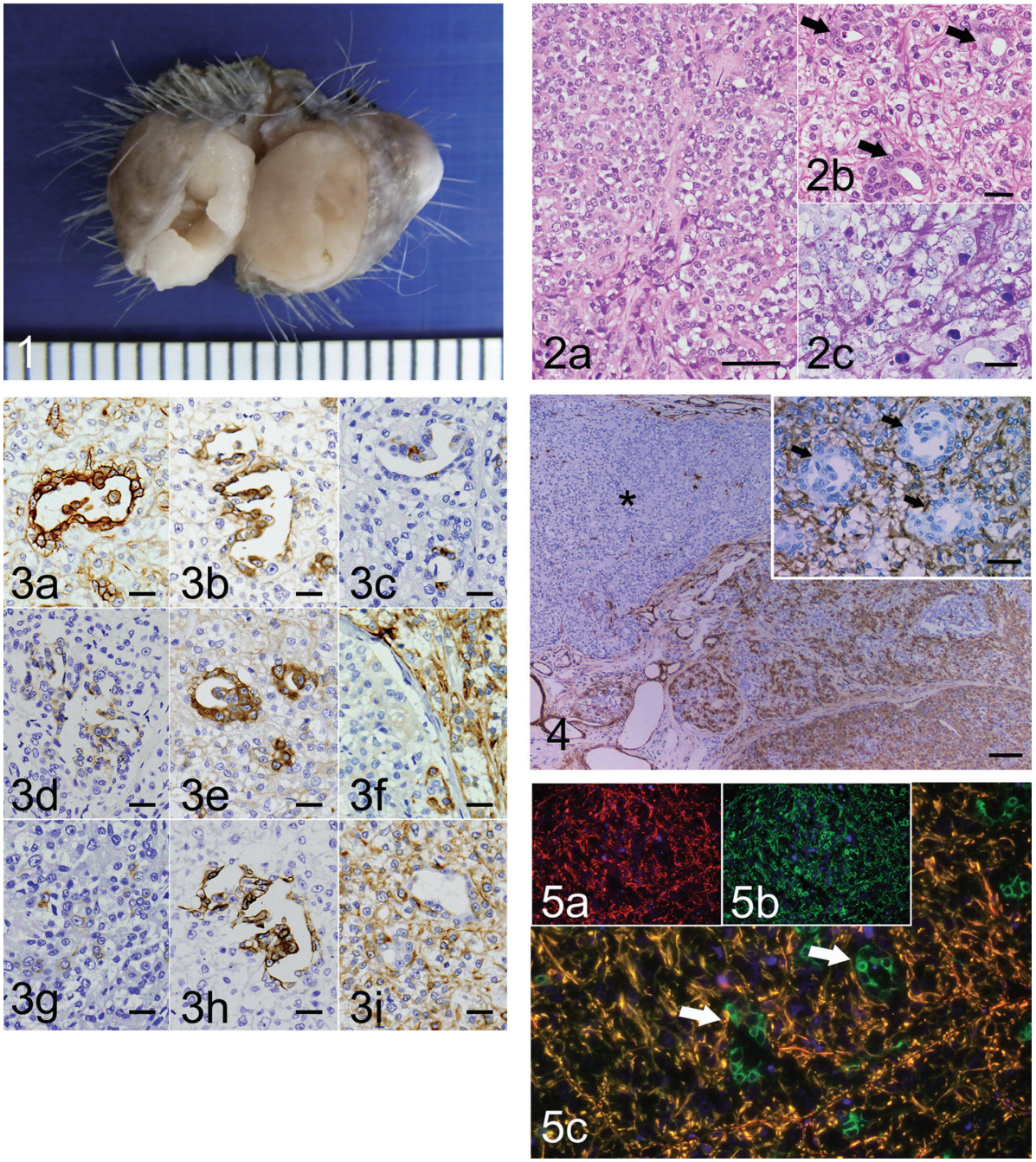

Fig. 1. A cut surface of the formalin-fixed neoplastic mass (8×8×10 mm).

Fig. 2. Histological appearances of the tumor. H&E staining. a) The tumor mostly consists of round or polygonal neoplastic cells with clear or vacuolated cytoplasms. Bar=50 μm. b) Small tubular structures are scattered in the neoplasm (arrows). Bar=20 μm. c) Fine glycogenic granules are present in the cytoplasm of the clear neoplastic cells. PAS stain. Bar=20 μm.

Fig. 3. a) Immunostaining for pan-CK (AE1/AE3): positive neoplastic cells are diffusely distributed. All tubular structures are strongly positive. b) Immunostaining for pan-CK (CAM 5.2): most neoplastic cells are positive, and tubular structures are strongly positive. c) Immunostaining for pan-CK (KL1): positive neoplastic cells including tubular cells are scattered. d) Immunostaining for CK-7: some of the neoplastic cells forming tubular structures are positive. e) Immunostaining for CK-8: most neoplastic cells are positive. Tubular structures are strongly positive. f) Immunostaining for CK-14: some areas are strongly positive. g) Immunostaining for CK-15: a few clear or vacuolated neoplastic cells are weakly positive. h) Immunostaining for CK-18: tubular structures are positive. i) Immunostaining for vimentin: most neoplastic cells, except tubular structures, are positive. Bar= 20 μm.

Fig. 4. Immunostaining for α-SMA. Tubular structures are negative (inset, Bar=20 μm). The neoplastic cells are mostly positive for α-SMA; however, neoplastic cells in a nodule (*) are barely positive. Bar=100 μm.

Fig. 5. Double-labeled immunofluorescence microscopy. Nuclei are colored blue with 4,6-diamino-2-phenylindole. a) Red fluorescence indicates vimentin immunostaining. b) Green fluorescence indicates pan-CK (AE1/AE3) immunostaining. c) Merge image. Yellow color indicates colocalized pan-CK and vimentin immunoreactivity. None of the tubular structures are positive just for vimentin (arrows).

Table 2. Results of Special Stains and Immunostains of Normal Skin Tissues and the Tumor[a]

		Normal tissues						Tumor
		Epidermis	Inner root sheath	Outer root sheath	Sebaceous gland	Apocrine gland	Follicular papilla	
Special stains								
PAS		–	–	+	–	–	–	+
Sudan III		–	–	–	+	–	–	–
Immunostains								
Antibody	Clone							
Cytokeratin	AE1/AE3	+	+	+	–	+	–	+ (>80%)[h]
Cytokeratin	KL1	+[b]	+	–	–	–	–	+ (1–10%)
Cytokeratin	CAM 5.2	–	+	–	–	+	–	+ (1–10%)
Cytokeratin 7	OC–TL 12/30	–	–	–	–	+	–	+ (<1%)
Cytokeratin 8	Ks8.7	–	–	+	–	+	–	+ (>75%)
Cytokeratin 14	LL002	+[c]	+	+	+	+[g]	–	+ (>30%)
Cytokeratin 15	CBL272	–	–	+[f]	–	–	–	+ (<1%)
Cytokeratin 18	Ks 18.4	–	–	–	–	+	–	+ (1–5%)
Cytokeratin 20	Ks 20.8	–[d]	–	–	–	–	–	–
Vimentin	V9	–[e]	–	–	–	–	+	+ (>80%)
α–SMA	1A4	–	–	–	–	+[g]	–	+ (>75%)
S–100a	polyclonal	–	–	–	–	–	–	+ (10–20%)
NSE	NSE–1G4	–	–	–	–	–	–	–
PGP 9.5	13C4	–	–	–	–	–	–	+ (10–20%)
Melan A	A103	–	–	–	–	–	–	–
MHC class II	TAL.1B5	–	–	–	–	–	–	±

[a] – = negative; + = positive. [b] Basal cell layer is negative. [c] Granular and horny cell layers are negative. [d] Merkel cells are positive. [e] Langerhans cells are positive. [f] Intermediate region is positive. [g] Only myoepithelial cells show a positive reaction. [h] The percentage indicates the proportion of the estimated positive cells in the tumor cells.

ever, in the present tumor, many neoplastic cells expressed α-SMA. Because actin is the predominant component of contractile microfilaments, which are responsible for cell motility and transport, α-SMA might play a role in cell migration and/or morphologic changes in the lower portion of the follicle during the hair cycle[9]. In mouse experiments *in vitro*, cells were found to express nestin in the bulge area and differentiated into neurons, glial cells, keratinocytes, smooth muscle cells and melanocytes[9,10]. These studies suggest that cells in the canine bulge area can differentiate into α-SMA-positive cells. Additionally, in normal canine skin, myoepithelial cells of the apocrine sweat gland are positive for α-SMA as well as CK, indicating that some α-SMA-positive neoplastic cells in the present case might have differentiated into myoepithelial cells. Apocrine sweat gland tumors with prominent proliferation of the myoepithelium component are considered to be a differential diagnosis due to their high positivity for α-SMA. However, most neoplastic cells also expressed vimentin, which ruled out apocrine sweat gland neoplasms. Moreover, the divergent differentiation of the neoplastic cells in this case is not seen in other cutaneous tumors, including complex apocrine sweat gland neoplasms.

In a previous report of clear cell adnexal carcinoma, tumor cells were positive for CK-8 and CK-18[6], thus suggesting they differentiated into the outer root sheath and apocrine sweat gland[6]. Other reports have shown that clear cell adnexal carcinoma is negative for α-SMA[4–6]. However, the present tumor was positive for CK-8, CK-18, pan-CK (KL1), CK-14 and α-SMA. Additionally, the present tumor most likely differentiated into the inner root sheath, outer root sheath and apocrine sweat gland (glandular epithelial cell and myoepithelial cell). This indicates that clear cell adnexal carcinoma has multilineage potential and differentiates into cutaneous adnexa and that this differentiation trend and the extent are different in each neoplasm.

Recently, CK-15, CK-19, CD34 and CD200 have been shown to be human follicular stem cell markers[6,11,12]. Canine hair follicles are reported to be more similar to human hair follicles than those of mice when considering their architecture and size[12]. Presumably, canine hair follicles could have stem cell machinery analogous to human hair follicles[12]. CK-15 and CD34 have been used as canine follicular stem cell markers in a previous study[12]. In the present case, CK-15 was positive in only 1% of neoplastic cells, regardless of the fact that clear cell adnexal carcinoma is suspected to be a neoplasm derived from follicular stem cells. Additionally, in normal skin, the intermediate region of the outer root sheath was positive for CK-15 (Table 2). In

this study, it is clear that the neoplastic cells probably differentiated into several adnexa.

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References

1. Gross TL, Ihrke PJ, Walder EJ, and Affolter VK. Skin Diseases of the Dog and Cat. 2nd ed, Blackwell Publishing, Oxford, U.K. 2005.
2. Sharif M and Reinacher M. Clear cell trichoblastomas in dogs. J Vet Med. **A53**: 352–354. 2006.
3. Nibe K, Uchida K, Itoh T, and Tateyama S. A case of canine apocrine sweat gland adenoma, clear cell variant. Vet Pathol. **42**: 215–218. 2005.
4. Mikaelian I and Wong V. Follicular stem cell carcinoma: histologic, immunohisitochemical, ultrastructural, and clinical characterization in 30 dogs. Vet Pathol. **40**: 433–444. 2003.
5. Schulman FY, Lipscomb TP, and Atkin TJ. Canine cutaneous clear cell adnexal carcinoma: histopathology, immunohistochemistry, and biologic behavior of 26 cases. J Vet Diagn Invest. **17**: 403–411. 2005.
6. Yasuno K, Nishiyama S, Suetsugu F, Ogihara K, Madarame H, and Shirota K. Cutaneous clear cell adnexal carcinoma in dog: special reference to cytokeratin expression. J Vet Med Sci. **71**: 1513–1517. 2009.
7. Kozaki M, Nakamura Y, Iguchi M, Kano R, Watanabe S, Fujiwara K, and Hasegawa A. Immunohistochemical analysis of cytokeratin expression in dog skin. J Vet Med Sci. **63**: 1–4. 2001.
8. Kato K, Uchida K, Nibe K, and Tateyama S. Immunohistochemical studies on cytokeratin 8 and 18 expressions in canine cutaneous adnexus and their tumors. J Vet Med Sci. **69**: 233–239. 2006.
9. Mancuso M, Leonardi S, Tanori M, Pasquali E, Pierdomenico M, Rebessi S, Majo VD, Covelli V, Pazzaglia S, and Saran A. Hair cycle-dependent basal cell carcinoa tumorigenesis in $Ptc1^{neo67/+}$ mice exposed to radiation. Cancer Res. **66**: 6606–6614. 2006.
10. Amoh Y, Li L, Katsuoka K, Penman S, and Hoffman RM. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci USA. **102**: 5530–5534. 2005.
11. Kloepper JE, Tiede S, Brinckmann J, Reinhardt DP, Meyer W, Faessler R, and Paus R. Immunophenotyping of the human bulge region: the quest to define useful in situ markers for human epithelial hair follicle stem cells and their niche. Exp Dermatol. **17**: 592–609. 2008.
12. Kobayashi T, Shimizu A, Nishifuji K, Amagai M, Iwasaki T, and Ohyama M. Canine hair-follicle keratinocytes enriched with bulge cells have the highly proliferative characteristic of stem cells. Vet Dermatol. **20**: 338–346. 2009.