NOTE Pathology

Cutaneous Clear Cell Adnexal Carcinoma in a Dog: Special Reference to Cytokeratin Expression

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ABSTRACT. A 5-year-old, male Bichon-Frise dog presented with a cutaneous mass in the basal region of the auricle. Histologically, the cutaneous neoplasm was comprised of lobules with solid cellular proliferation separated by thin fibrous septa. Neoplastic cells varied in size, with moderate to abundant amounts of PAS-positive cytoplasm, large nuclei and prominent nucleoli. Immunohistochemical examinations showed that tumor cells were positive for pan-cytokeratin (CK) (AE1/AE3 and CAM5.2), CK8 and CK18, but negative for pan-CK (KL1), CK7, CK14, CK16 and CK20. Double-labeled immunofluorescence testing indicated that neoplastic cells frequently co-expressed CK and vimentin, suggesting divergent differentiation of tumor cells. Based on these findings, the tumor was diagnosed as canine clear cell adnexal carcinoma.

KEY WORDS: canine, cytokeratin, immunohistochemistry, neoplasm, skin.

Malignant cutaneous tumors in dog with clear or vacuolated cytoplasm are uncommon, and include balloon cell melanoma, clear cell basal carcinoma, sebaceous carcinoma and clear cell adnexal carcinoma [3, 4, 12]. Canine cutaneous clear cell adnexal carcinoma was first proposed by Schulman et al. [12], and seems to be the same neoplasm known as clear cell hidradenocarcinoma or follicular stem cell carcinoma [12]. Clear cell adnexal carcinoma is a rare malignant neoplasm characterized by divergent differentiation into cutaneous adnexa, such as apocrine sweat glands and hair follicles. Clear cell adnexal carcinoma has been reported in dogs with a mean age of 6.9 years, with no apparent predilections for breed, sex or site [4, 9, 12]. This neoplasm shows a moderate prognosis following surgical excision, but infrequent recurrence and metastasis to local lymph nodes has been reported [12]. The origin of this tumor is likely to be follicular stem cells, as some tumor cells immunohistochemically show double-positivity for cytokeratin (CK) and vimentin [9, 12].

CKs are classified according to molecular weight and isolectric points, and are numbered from 1 to 20 [1]. Examination of CK isoforms in clear cell adnexal carcinoma may be useful to identify divergent differentiation and tumor origin, as expression of CK isoforms differs among the epithelial cells of various cutaneous adnexa. We present herein a case of canine clear cell adnexal carcinoma and describe the immunohistological properties, particularly in terms of CK isoform expressions.

A 5-year-old, male Bichon-Frise dog was brought in with a red papule approximately 8 mm in diameter (Fig. 1). The mass was located on the skin at the basal region of the left auricle, and no swelling or inflammatory signs were apparent in surrounding tissues. Six months later, cytoscreening was performed and squamous cell carcinoma or malignant trichoepithelioma was suspected by a diagnostic laboratory. The tumor mass was then surgically excised and submitted to our laboratory for histopathological examination. On cut section, the mass was grey-white and well-defined, with partial hemorrhage. During the 11 months since the surgical excision, no local recurrence or metastasis has been recognized.

Excised tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained using hematoxylin and eosin (HE), Periodic acid-Schiff (PAS), Sudan III and Fontana-Masson silver impregnation. Immunohistochemical staining was performed by immunoenzyme polymer method using the primary antibodies shown in Table 1. Peroxidase-conjugated anti-mouse (Histofine Simple Stain MAX-PO(M); Nichirei, Tokyo, Japan) or peroxidase-conjugated anti-rabbit (Histofine Simple Stain MAX-PO(R); Nichirei) immunoglobulin (IgG) were used as secondary antibodies. These immunohistochemical stains were incubated with diaminobenzidine and counterstained with Mayer’s hematoxylin. Nine samples of canine trichoblastoma were used for immunohistochemical comparison and intact skin samples taken from several dogs were used as normal control tissues. Neoplastic cells in trichoblastomas were divided into palisade/basaloid cells and spindle/elongated cells by their histological features. To determine co-expression of CK and vimentin in neoplastic cells, double-labeled immunofluorescence testing was performed using anti-CK (clone AE1/AE3) and anti-vimentin antibodies as primary antibodies. In this assay, affinity-purified goat anti-mouse IgG fluorescein isothiocyanate (FITC) (EY Labora-
Fig. 1. The neoplastic mass in the skin of the basal region of the left auricle.

Fig. 2. The cutaneous neoplasm is well-demarcated and densely cellular. The mass is comprised of variably sized lobules and a large cystic area. Hematoxylin and Eosin (HE) stain. Bar=1,000 μm.

Fig. 3. a) The neoplastic lobule with a follicular papilla-like structure in the periphery of the lobule (arrow). HE. Bar=50 μm. b) Neoplastic cells vary in size, showing a moderate to abundant amount of eosinophilic cytoplasm with large nuclei and nucleoli. Spindle cells and multinucleated cells are also present (arrow). HE. Bar=25 μm. c) The majority of tumor cells includes Periodic acid-Schiff (PAS)-positive fine granules in the cytoplasm. PAS. Bar=15 μm.
Histologically, the cutaneous neoplasm was well-demarcated and densely cellular. The mass was comprised of variably sized lobules separated by thin fibrous bands, and a large cystic structure was also present (Fig. 2). The cyst wall was composed of layers of neoplastic cells and the cyst was filled with blood and defoliated tumor cells. Neoplastic cells varied in size, and ranged in shape from round to polygonal to spindle-shaped. Moderate to abundant amounts of pale eosinophilic cytoplasm were present along with a large nucleus and prominent nucleolus (Fig. 3a). Multinucleated tumor cells were occasionally observed, with some including more than ten nuclei (Fig. 3b). Most tumor cells had vacuolated or clear cytoplasm, which was negative for Sudan III and positive for PAS with a fine granular appearance (Fig. 3c). Most PAS-positive granules were diastase-labile, indicating glycogen. Fontana-Masson staining did not identify melanin in tumor cells and no neuroendocrine granules were identified in the cytoplasm ultrastructurally. Occasionally, clusters of small, dark, round to spindle-shaped nuclei suggestive of the papilla of a hair follicle were observed adjacent to neoplastic lobules (Fig. 3a). Mitotic rate was approximately 4 per 10 high-powered field.

The results of immunohistochemical examinations are summarized in Table 2. Neoplastic cells were constantly positive for pan-CK (AE1/AE3 and CAM 5.2), and also CK 8 and CK18, but negative for other CK markers (Fig. 4). Neoplastic cells positive for CAM 5.2 were often spindle-shaped (Fig. 4c), although round or polygonal-shaped cells with vacuoles were negative. A monoclonal antibody, CK19 (Ks19.1), did not react with any cutaneous cells from normal dogs or the present case. Double-labeled immunofluorescence testing revealed CK-vimentin double-positive cells among the neoplastic cells, particularly within the spindle-shaped cell population (Fig. 5). Follicular papilla-like structures (follicular papillary mesenchymal bodies) were negative for CK, but positive for vimentin. Several S-100a-positive tumor cells were present in peripheral areas of neoplastic lobules (Fig. 4k), but no neuron specific enolase (NSE)-positive cells were seen within the tumor mass. MHC class II-positive cells were also present within the neoplastic lobules, and formed cell processes, indicating dendritic cells (Fig. 4l). Results of immunohistochemical examinations of trichoblastomas and normal tissues are also

tories, San Mateo, California, U.S.A.) and affinity-purified goat anti-mouse IgG Rhodamine (Chemicon, Temecula, California, U.S.A.) were used to label anti-CK and antivimentin antibody, respectively. In addition, a part of the formalin-fixed tissue specimen was cut into 1-mm³ cubes, re-fixed in 2.5% glutaraldehyde, post-fixed in 1% OsO4 and embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, then examined using a JEOL 1210 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Table 1. Immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Antibody source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin</td>
<td>AE1/AE3</td>
<td>1:50</td>
<td>trypsin</td>
<td>Dako</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>KL1</td>
<td>1:100</td>
<td>MW</td>
<td>Immunotech</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>CAM 5.2</td>
<td>prediluted</td>
<td>proteinase K</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>OC-TL 12/30</td>
<td>prediluted</td>
<td>proteinase K</td>
<td>Dako</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Ks 8.7</td>
<td>prediluted</td>
<td>MW</td>
<td>Progen Biotechnik</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>LL002</td>
<td>1:100</td>
<td>MW</td>
<td>Serotec</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>LL025</td>
<td>1:50</td>
<td>MW</td>
<td>Thermo</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Ks 18.4</td>
<td>prediluted</td>
<td>proteinase K</td>
<td>Progen Biotechnik</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Ks 19.1</td>
<td>prediluted</td>
<td>proteinase K</td>
<td>Progen Biotechnik</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>Ks 20.8</td>
<td>prediluted</td>
<td>proteinase K</td>
<td>Dako</td>
</tr>
<tr>
<td>Vimentin</td>
<td>V9</td>
<td>1:25</td>
<td>MW</td>
<td>Dako</td>
</tr>
<tr>
<td>o-SMA</td>
<td>IA4</td>
<td>1:50</td>
<td>NT</td>
<td>Dako</td>
</tr>
<tr>
<td>S-100a</td>
<td>polyclonal</td>
<td>1:200</td>
<td>MW</td>
<td>Dako</td>
</tr>
<tr>
<td>NSE</td>
<td>NSE-1G4</td>
<td>1:100</td>
<td>MW</td>
<td>Zymed</td>
</tr>
<tr>
<td>Melan A</td>
<td>A103</td>
<td>1:50</td>
<td>MW</td>
<td>Dako</td>
</tr>
<tr>
<td>MHC class II</td>
<td>TAL.1B5</td>
<td>1:100</td>
<td>MW</td>
<td>Dako</td>
</tr>
</tbody>
</table>

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, California, U.S.A.; Zymed, California, U.S.A.

Fig. 4. Immunostaining of tumor tissues. Immunostaining for cytokeratin (CK). a) CK clone AE1/AE3. b) CK clone KL1. c) CK clone CAM5.2. The majority of positive cells is spindle-shaped. d) CK 7. e) CK 8. f) CK 14. g) CK 16. h) CK 18. i) CK 20. Most neoplastic cells are positive for CK clone AE1/AE3, CAM 5.2, CK8 and CK18. Many neoplastic cells are also positive for vimentin. j) Vimentin. k) S-100a. Several positive cells are observed peripherally in neoplastic lobules (arrow). l) MHC class II. Dendritic cells forming cell processes are observed within the tumor. Bar=25 µm.

Fig. 5. Double-labeled immunofluorescence microscopy of tumor cells. Green color indicates positive staining for CK clone AE1/AE3. Red color shows positive staining for vimentin. Nuclei are colored blue with 4,6-diamino-2-phenylindole. a) CK clone AE1/AE3. FITC. b) Vimentin. Rhodamin. c) Merge. Yellow or orange colored cells show co-expression of CK and vimentin.
K. YASUNO ET AL.

described in Tables 2 and 3. Finally, the cutaneous tumor was diagnosed as clear cell adnexal carcinoma on the basis of morphological and immunohistochemical characterization. Differential diagnoses for canine clear cell adnexal carcinoma include balloon cell melanoma, clear cell basal carcinoma and sebaceous carcinoma, due to various morphological similarities. On immunohistochemical examination, CK-positive results rule out melanocytic neoplasms. Basal cell carcinoma is reportedly positive for KL1 and CK14 [5], which were negative in our case. Sebaceous carcinoma was also excluded based on histological and immunohistochemical properties.

In the present case, cellular differentiation into a hair follicle was suggested from the presence of follicular papilla-like structures and expression of CK8. CK8 is expressed in the outer root sheath cells, but also expressed in the normal sweat gland [6, 8, 10] (Table 3). The outer root sheath cell includes PAS-positive granules in the cytoplasm, which was also seen in our case. These results suggest differentiation of neoplastic cells into outer root sheath cells, but we did not detect any differentiation to inner root sheath cells. Inner root sheath cells are positive for CK16 and KL1, but these were negative in our case (Table 2). Formation of subtle tubular structures suggesting differentiation into a sweat gland has been reported in other cases of clear cell adnexal

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Tumor</th>
<th>Trichoblastoma(n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin</td>
<td>AE1/AE3</td>
<td>(+ (&gt;75%)&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>(+ (&gt;10%)&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>KL1</td>
<td>–</td>
<td>+ (0–10%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>CAM 5.2</td>
<td>(+ (&gt;50%))</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 7</td>
<td>OC-TL 12/30</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>Ks 8.7</td>
<td>(+ (&gt;75%))</td>
<td>(+ (&gt;10%))</td>
</tr>
<tr>
<td>Cytokeratin 14</td>
<td>LL002</td>
<td>–</td>
<td>+ (&gt;10%)</td>
</tr>
<tr>
<td>Cytokeratin 16</td>
<td>LL025</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 18</td>
<td>Ks 18.4</td>
<td>(+ (&gt;75%))</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 20</td>
<td>Ks 20.8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Vimentin</td>
<td>V9</td>
<td>(+ (&gt;50%))</td>
<td>–</td>
</tr>
<tr>
<td>α-SMA</td>
<td>1A4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S-100a</td>
<td>polyclonal</td>
<td>+ (1–10%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>NSE</td>
<td>NSE-1G4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Melan A</td>
<td>A103</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MHC class II</td>
<td>TAL.1B5</td>
<td>±</td>
<td>± (&lt;1%)</td>
</tr>
</tbody>
</table>

– = negative; ± = few positive cells within tumor tissues; + = positive. <sup>a</sup> The percentage indicate the estimated proportion of the positive cells.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Normal tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin</td>
<td>AE1/AE3</td>
<td>Epidermis</td>
</tr>
<tr>
<td>Cytokeratin</td>
<td>KL1</td>
<td>+</td>
</tr>
<tr>
<td>Cytokeratin</td>
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<td>–</td>
</tr>
<tr>
<td>Cytokeratin 7</td>
<td>OC-TL 12/30</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 8</td>
<td>Ks 8.7</td>
<td>–</td>
</tr>
<tr>
<td>Cytokeratin 14</td>
<td>LL002</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Cytokeratin 16</td>
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<td>+</td>
</tr>
<tr>
<td>Cytokeratin 18</td>
<td>Ks 18.4</td>
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<td>Cytokeratin 20</td>
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<td>–&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vimentin</td>
<td>V9</td>
<td>–&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>α-SMA</td>
<td>1A4</td>
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<tr>
<td>S-100a</td>
<td>polyclonal</td>
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</tr>
<tr>
<td>NSE</td>
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<tr>
<td>Melan A</td>
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<tr>
<td>MHC class II</td>
<td>TAL.1B5</td>
<td>–&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

a) – = negative; + = positive, b) basal cell layer was negative, c) granular and horny cell layers were negative, d) Merkel cells were positive, e) Langerhans cells were positive, f) melanocytes were positive, g) only myoepithelial cells showed positive reaction.
carcinoma [9, 12], but was not found in our case. The large cystic structure observed in the present case may suggest differentiation into a tubular structure. Moreover, expression of CK18 suggested differentiation into sweat gland, as CK18 expression is specifically observed in the normal sweat gland [6, 8, 10] (Table 3). In contrast, CK7 expression is also reportedly specific to normal sweat glands [2], but our case was negative for CK7 (Table 2). Combining these findings, these features indicate that neoplastic cells in our case showed inconclusive differentiation to sweat gland cells, because of the positivity for CK18. Differentiation to melanocytes has also been suggested in the majority of clear cell adnexal carcinomas on the grounds of positivity with Fontana-Masson staining and melan-A [9, 12], both of which were negative in our case. Tumor differentiation to neuroendocrine cells has also been suggested on the basis of ultrastructural findings in several cases [9], but we did not detect neuroendocrine granules. A lack of CK20 expression excluded differentiation of the neoplasm to Merkel cells.

The origin of clear cell adnexal carcinoma is considered to involve follicular stem cells, with tumor cells later showing divergent differentiation into cutaneous adnexa [9, 12]. In mice, follicular stem cells can form hair follicles, sweat gland, sebaceous gland and epidermis [11]. As in this case, divergent differentiation to hair follicle and sweat gland, and co-expression of CK and vimentin strongly suggest that the tumor was likely to have originated as a follicular stem cell. Recently, follicular stem cell markers such as CK15, CK19, CD34 and CD200 have been reported in humans [7], but no precise markers have been described for canine follicular stem cells. Further research is required to clarify the origins of canine clear cell adnexal carcinoma.

Trichoblastoma must be considered as a differential diagnosis, given the lower proportion of the clear cell population in the present case as compared with previous cases [9, 12], and the absence of tubular structures. Some types of trichoblastoma, including granular cell or clear cell trichoblastoma, show presence of a PAS-positive clear cell population as a clear cell adnexal carcinoma [3, 4, 13]. Clear cell trichoblastoma is a rare variant for which only a single case in humans and 2 cases in dogs have been reported [7], but no precise markers have been described for canine follicular stem cells. Further research is required to clarify the origins of canine clear cell adnexal carcinoma.

This paper is the first report of detailed immunohistochemical examination for cytokeratin expression in a case of canine clear cell adnexal carcinoma.

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