

Lymphangiosarcoma with bone formation of the auricle in a dog

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(Received 22 December 2014/Accepted 30 January 2015/Published online in J-STAGE 12 February 2015)

ABSTRACT. A 12-year-old mixed-breed neutered female dog was referred with cutaneous tumors at the left auricle. Histologically, the cutaneous tumor located in the dermis comprised numerous clefts and cavernous channels lined by neoplastic endothelial cells with no erythrocytes. Bone tissue without direct contact with neoplastic cells was seen in the well-developed stromal connective tissue. The neoplastic endothelial cells exhibited mild to moderate atypia. Immunohistochemically, neoplastic cells were positive for vimentin and negative for cytokeratin and factor VIII-related antigen. Basement membrane around the neoplastic lumens was positive for laminin in a linear or granular pattern. Ultrastructural examination revealed discontinuous basement membrane beneath the tumor cells. Histopathological features of this case were consistent with lymphangiosarcoma, and stromal ossification was characteristic.

KEY WORDS: bone, canine, tumor pathology

doi: 10.1292/jvms.14-0682; *J. Vet. Med. Sci.* 77(6): 739–742, 2015

Lymphangiosarcoma is a rare malignant cutaneous tumor that derives from the lymphatic endothelium in humans and domestic animals [1–5, 8, 9, 11–13]. Only a small number of case reports have described canine lymphangiosarcoma [1, 4, 9, 11, 14]. In dogs, lymphangiosarcoma tends to localize in the subcutis along the ventral midline and limbs, with major reported locations of the inguinal region, cervical region, hind limb and forelimb [1, 4, 9, 11, 14].

We recently encountered a unique case of canine lymphangiosarcoma with bone formation in the auricle. We describe herein the morphological and immunohistochemical findings of a case of lymphangiosarcoma of the auricle in a dog.

A 12-year-old mixed-breed neutered female dog developed cutaneous masses at the left auricle. The dog had a history of bilateral chronic pruritic otitis externa. The largest mass at the tip of the auricle was found by the dog's owner 1.5 years before resection of the auricle and had become gradually enlarged since first discovery. The mass in the region of the tip of the auricle had slowly grown to 28 × 25 mm, but no swelling was apparent in surrounding tissues (Fig. 1). Two millet-sized daughter nodules developed on the postauricular skin during the period of primary tumor development. Resection of the auricle was performed, and the specimen was submitted to our laboratory for histopathological examination. The largest mass was soft on palpation, but difficult to transect due to presence of a calcified lesion within the center of the mass. Effusion of a clear serous fluid

was noted from the cut surface. During the 14 months since surgical excision, no local recurrence or metastasis has been recognized.

The mass was fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE), alcian blue (pH 2.5), periodic acid-Schiff (PAS) and Watanabe's silver impregnation. Surface decalcification of paraffin-embedded sections for HE staining was performed by placing tissue blocks in Plank-Rychlo's solution (0.3 M aluminium chloride, 3% HCl and 5% formic acid) for 20 min at room temperature. Immunohistochemistry (IHC) was performed by the 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 anti-rabbit (Histofine Simple Stain MAX-PO (R); Nichirei) immunoglobulin (Ig) G was used as secondary antibodies. Immunofluorescence testing was performed using anti-laminin antibody (Table 1). After blocking with 4% Block Ace™ (Snow Brand Milk Products, Sapporo, Japan) for 10 min at room temperature, dewaxed sections were incubated with anti-laminin antibody. After washing with PBS, sections were stained with Alexa Fluor 488 conjugated anti-rabbit IgG (Invitrogen, Tokyo, Japan). Fluorescence was analyzed using a FSX100 fluorescence microscope (Olympus, Tokyo, Japan). In addition, part of the formalin-fixed tissue specimen was cut into 1-mm³ cubes, re-fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium acid and embedded in Epon. Ultra-thin sections were double-stained with uranyl acetate and lead citrate and then examined using a transmission electron microscope (JEOL 1210; JEOL, Tokyo, Japan) at 80 kV.

Histologically, the largest infiltrative mass at the tip region was located in the dermis. The mass comprised numerous irregular lumens or slit-like spaces with no erythrocytes and lined by plump or spindle-shaped neoplastic cells (Fig. 2). Neoplastic cells exhibited mild to moderate atypia and pleo-

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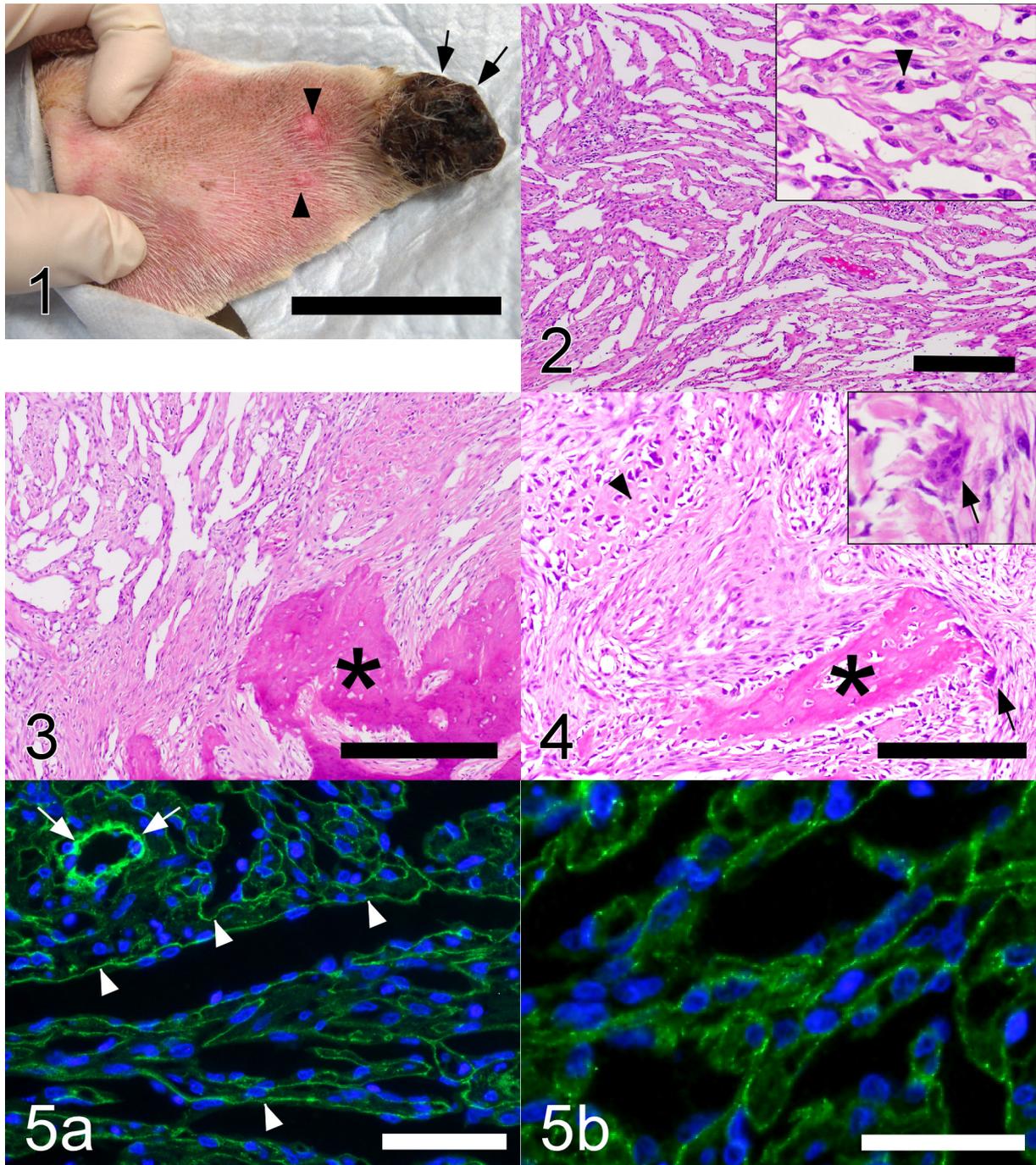


Fig. 1. The cutaneous mass (arrows) in the region of the tip of the left auricle. Two millet-sized daughter nodules (arrowheads) were present on postauricular skin. Bar=50 mm.

Fig. 2. The mass was located in the dermis and comprised numerous clefts and cavernous channels lined by neoplastic endothelial cells with no erythrocytes. *Inset*: Mitotic figure in the lining cells (arrowhead). Hematoxylin and eosin (HE) stain. Bar=300 μ m.

Fig. 3. Bone formation (asterisk) in the well-developed stroma separated from neoplastic cells. HE stain, decalcified tissue. Bar=200 μ m.

Fig. 4. Osteoclast-like giant cells (arrows) and osteoblasts were frequently found around the bone tissue (asterisk). Osteoid formation (arrowhead) occurred near the bone tissue. *Inset*: Osteoclast-like giant cells. HE stain, decalcified tissue. Bar=200 μ m.

Fig. 5. Immunofluorescence (IF) for laminin. Green color indicated positive staining for laminin. Nuclei were colored blue with 4,6-diamino-2-phenylindole. (a) Laminin expression was linear or granular around neoplastic vascular channels (arrowheads). The wall of non-neoplastic vessel showed intense positive staining for laminin (arrows). Bar=50 μ m. (b) High magnification view of IF for laminin. Laminin expression was granular around neoplastic vascular channels. Bar=30 μ m.

Table 1. Primary antibodies and immunostaining protocol in the current study

Antibody ^{a)}	Clone	Dilution	Source	Antigen retrieval ^{b)}
Pan-CK	AE1/AE3	1:50	Dako Denmark A/S., Glostrup, Denmark	MW, 95°C, 10 min
Vimentin	SP20	1:100	Nichirei Corp., Tokyo, Japan	MW, 95°C, 10 min
Factor VIII-related antigen	Polyclonal	prediluted	Dako Denmark A/S., Glostrup, Denmark	pepsin, 37°C, 20 min
SMA	1A4	1:100	Dako Denmark A/S., Glostrup, Denmark	No treatment
Ki67	MIB-1	1:100	Dako Denmark A/S., Glostrup, Denmark	AC, 121°C, 20 min
Laminin	Polyclonal	1:100	Progen Biotechnik., Heidelberg, Germany	pepsin, 37°C, 20 min

a) CK=cytokeratin, b) MW=microwave, citrate buffer (PH6.0); AC=autocleave, citrate buffer (PH6.0); Pepsin=0.4% pepsin (Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

morphism, varying in size. The mitotic activity of neoplastic cells was mild, at 2 mitoses per 10 high-power fields. Localized bone tissue without direct contact with neoplastic cells and cartilage of the auricle was seen in the stromal connective tissue (Fig. 3). The bone tissue did not show lamellar structure and was irregular in shape, approximately 6 mm in maximum length. Osteoclast-like giant cells and polyhedral osteoblasts were frequently found on the surface of the bone tissue. Fibrous stroma around the bone contained irregular osteoid structures (Fig. 4). Alcian blue staining demonstrated no cartilaginous components in and around the bone tissue. Marked necrosis and superficial ulceration of the epidermis with infection by Gram-positive cocci were noted at the tip of the ear. Inflammatory cells, mainly comprising lymphocytes and plasma cells, were scattered throughout the tumor and adjacent soft tissues. Histopathologically, daughter nodules exhibited similar features to the largest mass, but without formation of bone tissue.

Silver staining demonstrated that most neoplastic cells proliferated along reticular fibers. PAS-stained sections confirmed discrete basement membrane around intratumoral non-neoplastic capillary vessels, but results were unclear around neoplastic vessels. Basement membrane also showed staining with anti-laminin antibody. Laminin staining was linear or granular around the neoplastic lumens or slit-like spaces (Fig. 5).

Neoplastic cells showed diffuse expression of vimentin, but no positive labeling for cytokeratin (CK) AE1/AE3. Factor VIII-related antigen was intensely positive in normal endothelial cells of both blood and lymphatic vessels, but neoplastic cells showed no labeling for factor VIII-related antigen. Smooth muscle actin-positive pericyte layers were not seen in the structures of any lumens or slit-like spaces. The proliferation rate, evaluated as the percentage of Ki67-positive cells in 5 high-power fields (including approximately 1,000 cells), was 10.7%.

Ultrastructural examination revealed empty clefts devoid of blood cells and lined by neoplastic cells with oval nuclei and a paranuclear zone rich in intermediate filaments. There were no pericytes around the neoplastic vasculatures. Weibel-palade bodies were not found in the neoplastic cells examined. Basement membrane was apparent, but partly unclear beneath the tumor cells, of which findings might be consistent with those in immunofluorescence for laminin (Fig. 6).

The differential diagnosis of hemangiosarcoma and

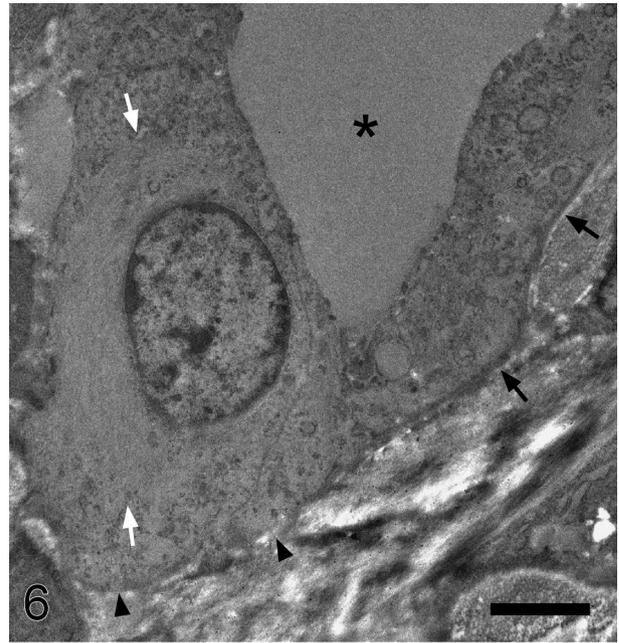


Fig. 6. Ultrastructural examination revealed empty clefts (asterisk) devoid of blood cells, lined by neoplastic cells with oval nuclei and a paranuclear zone rich in intermediate filaments (white arrows). Basement membrane beneath the neoplastic cells was detected (black arrows), but it was partly unclear (arrowheads). Weibel-palade bodies were not present in the neoplastic cells. Bar=2 μ m.

lymphangiosarcoma is based on both immunohistochemical and morphological features. Recently, Halsey *et al.* [4] have shown usability of novel lymphatic endothelial cells markers; lymphatic vessel endothelial receptor-1 (LYVE-1) and prospero-related homeobox-gene-1 (PROX-1) for differential diagnosis of vascular tumors in dogs. However, in the present case, a lack or greatly reduced presence of erythrocytes, lymphoplasmacytic infiltration in the tumor, absence of pericytes and lack of Weibel-palade bodies in the neoplastic cells suggest the tumor to be a lymphangiosarcoma, not a hemangiosarcoma [3, 15]. Moreover, unclear or discontinuous basement membrane as seen by electron microscopy or PAS staining in the present case was consistent with previous descriptions of lymphangiosarcoma in animals [2, 3, 9–11]. The granular immunoreactivity for laminin around neoplastic vascular channels might indi-

cate discontinuous basement membrane in agreement with ultrastructural finding. According to some previous cases, distinguishing well-differentiated lymphangiosarcoma from lymphangiomatosis might be difficult. In our case, the presence of daughter nodules, mitotic activity and markedly infiltrative growth separated lymphangiomatosis from lymphangiosarcoma.

This is the first report of canine lymphangiosarcoma which occurred in the auricle. The trigger for the development of canine lymphangiosarcoma remains unclear in most cases. However, most lymphangiosarcomas in humans arise against a background of chronic lymphedema following mastectomy, trauma or irradiation [13]. In the present case, the dog had no history of chronic lymphedema at the tumor site. We suspect that chronic inflammation and trauma associated with pruritic otitis externa might have been involved in the oncogenesis in this case.

As stated above, the tumor was diagnosed as a lymphangiosarcoma. However, the case was not histologically typical, because of the accompanying intratumoral bone tissue. Bone formation has been reported in other soft tissue tumors, including one case of ossifying epithelioid hemangioendothelioma in humans [7]. To the best of our knowledge, bone formation has not been reported in any previous cases of lymphangiosarcoma in dogs or humans. In the present case, bone tissue in fibrous stroma separated from neoplastic cells indicated that stromal mesenchymal cells might differentiate into osteoblasts. The mechanisms and biological signals involved in extraskelatal bone formation are poorly understood. Imai *et al.* [6] reported that bone morphogenetic proteins 2, 4, 5 and 6 may play important roles in ectopic bone formation in human colon carcinoma. Analysis focused on these factors might thus provide valuable information regarding bone formation in the present case.

ACKNOWLEDGMENTS. We wish to thank Dr. Yoko Kakinuma and Ms. Yuka Isayama of Azabu University for their valuable assistance.

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