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NOTE

Pathology

Anaplastic large T-cell lymphoma in three black-tailed prairie dogs (*Cynomys ludovicianus*)

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ABSTRACT. Anaplastic large T-cell lymphoma (ALTCL) is rarely reported in domestic animals. Accordingly, the histopathological and immunohistochemical characteristics of ALTCL have not been well established in prairie dogs. The present report documents three cases in which prairie dogs were diagnosed with ALTCL arising in the subcutaneous tissue, oral mucosa or the mesenteric lymph nodes. Of the three cases, one was available for necropsy and the others were biopsy cases. Microscopically, moderate to large, pleomorphic neoplastic lymphocytes with ovoid to polygonal, bizarre-shaped nuclei, abundant cytoplasm and eosinophilic granules were seen in all cases. Immunohistochemical staining revealed membranous or cytoplasmic CD3 expression of the neoplastic lymphocytes. The neoplastic cells often had granzyme B-positive cytoplasmic granules. One of the prairie dogs with nodal ALTCL suffered systemic dissemination of the tumor and died suddenly. In the two biopsy cases, one animal died on the day of the biopsy examination and the other died six weeks after chemotherapy. ALTCL in prairie dogs displays a cytotoxic T cell phenotype and presumably carries a poor prognosis regardless of the anatomical type.

KEY WORDS: anaplastic large T-cell lymphoma, black-tailed prairie dog, granzyme B

Prairie dogs (*genus Cynomys*) are burrowing rodents that are native to the grasslands of North America. Of the five species of prairie dogs, the black-tailed prairie dog (*Cynomys ludovicianus*) is commonly kept as a pet in Japan. However, the population of prairie dogs in Japan has been gradually decreasing as a ban on their importation was put in place in 2003.

Lymphoma has rarely been reported in prairie dogs [3, 8]. Thus, there is limited information about lymphoma in prairie dogs and no optimal treatment for the condition has been established. A retrospective study performed by Thas and Garner reported that 13.2% of cases of neoplasia in prairie dogs involved tumors of hematolymphoid origin, including multicentric lymphoma (n=2), malignant round cell tumors (n=2), high-grade lymphoma of the liver and gall bladder (n=1), cutaneous lymphoma (n=1) and malignant thymoma (n=1) [8]. In addition, Miwa *et al.* described a case of multicentric lymphoma combined with systemic dissemination in a prairie dog [3]. The affected animal died of progressive respiratory failure despite supportive care and chemotherapy with cyclophosphamide.

Anaplastic large T-cell lymphoma (ALTCL) is a rare neoplasm of the hematopoietic system in domestic animals [9, 10]. ALTCL is characterized by the malignant proliferation of aberrant T cells and has a poor prognosis [9]. To the best of our knowledge, the immunohistochemical profile and biological behavior of ALTCL have not been well defined in prairie dogs. In the present study, we characterized the histopathological features of ALTCL in three prairie dogs.

Biopsy or necropsy tissue specimens were collected from three black-tailed prairie dogs that presented at Miwa Exotic Animal Hospital between 2010 and 2016. Details of these cases are described in Table 1. Routinely fixed, paraffin-embedded tissue blocks were cut into 4 μm sections. The sections were deparaffinized, rehydrated through a graded series of alcohols and stained with hematoxylin and eosin (HE).

Immunohistochemical analyses were performed using anti-CD3, CD79α, CD20 and CD56, ionized calcium-binding adapter molecule 1 (Iba-1), granzyme B, and Ki-67 antibodies. The patterns and intensity of the staining were compared with those seen in the internal positive and negative controls. Briefly, antigen retrieval was achieved by autoclaving the sections at 121°C for 10 min. Endogenous peroxidase was inactivated by treatment with 3% hydrogen peroxide in methanol at room temperature for 5 min. The

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Table 1. Clinicopathological data of the prairie dogs

Case No.	Age	Sex	Lesion location	Pathological diagnosis
1	8 years	M	Lumbar skin	Cutaneous ALTCL
2	10 years 6 months	F	Oral mucosa	Oral ALTCL
3	Not available	F	M-LN, SI, LI, liver, spleen, kidneys, adrenal gland, lungs	Nodal ALTCL

ALTCL, anaplastic large T cell lymphoma; M, male; F, female; M-LN, mesenteric lymph node; SI, small intestine; LI, large intestine.

Table 2. Antibodies used for the immunohistochemistry

Antibody	Clone	Type	Dilution	Manufacturer	Antigen retrieval
CD3		pAb rabbit	RTU	Dako Japan (Tokyo, Japan)	Heat, sodium citrate buffer (pH6)
CD20		pAb rabbit	1:400	Thermo Fisher Scientific (Waltham, MA, U.S.A.)	None
CD56	1G4	mAb mouse	1:150	LifeSpan BioSciences (Seattle, WA, U.S.A.)	Heat, sodium citrate buffer (pH6)
CD79α	HM57	mAb mouse	1:50	Dako Japan (Tokyo, Japan)	Heat, high pH target retrieval (pH9)
Granzyme B		pAb rabbit	1:100	Spring Bioscience (Pleasanton, CA, U.S.A.)	Heat, sodium citrate buffer (pH6)
Iba-1		pAb rabbit	1:500	Wako (Osaka, Japan)	Heat, sodium citrate buffer (pH6)
Ki-67	MIB-1	mAb mouse	RTU	Dako Japan (Tokyo, Japan)	Heat, sodium citrate buffer (pH6)

pAb, polyclonal antibody; mAb, monoclonal antibody; RTU, ready to use; IHC, immunohistochemistry.

sections were then blocked with 8% skimmed milk in Tris-buffered saline (TBS) at 37°C for 30 min, before being incubated with the primary antibodies at 4°C overnight. After being washed in TBS three times, the sections were incubated with Dako EnVision+ System horseradish peroxidase-labeled polymer secondary antibodies (Dako Japan, Tokyo, Japan) at 37°C for 40 min. The sections were washed three times in TBS, before being visualized using 0.05% 3,3′-diaminobenzidine and 0.03% hydrogen peroxidase in Tris-HCl buffer. The co-expression of CD3 and granzyme B was confirmed by immunofluorescent staining. The antigen retrieval procedure was performed as described above. Following incubation with the first primary antibody (anti-granzyme B) at 4°C overnight, the sections were incubated with the second primary antibody (anti-CD3) at 37°C for 60 min. After being washed three times in TBS with 1% tween 20 (TBST), they were incubated with Alexa 488-conjugated goat anti-mouse IgG (1:200, Invitrogen, OR, U.S.A.) and Alexa 594-conjugated donkey anti-rabbit IgG (1:200, Invitrogen) at room temperature for 1 hr. The co-expression of CD3 and granzyme B was analyzed using a laser-scanning confocal microscope (LSM700; Zeiss, Tokyo, Japan). The details of the antibodies and antigen retrieval methods are shown in Table 2. The mitotic count (MC) was manually counted per 10 high-power fields (HPF). The MIB-1 index was determined by manually counting the number of Ki-67-positive cells per total neoplastic cells. The specimens were reviewed by two Japanese College of Veterinary Pathologists-accredited veterinary pathologists (KU and JKC).

Case 1. An 8-year-old intact male prairie dog was presented with a 2-month history of a firm solitary mass in the lumbar region. The lesion gradually increased in size and markedly ulcerated at the time of presentation. Microscopically, the ulcerated lesion was accompanied by hemorrhaging and suppurative inflammation. The infiltrative proliferation of moderate to large pleomorphic neoplastic lymphocytes was seen from the dermis to the margins of the skeletal muscle. The neoplastic lymphocytes had ovoid to polygonal nuclei composed of dense and coarsely clumped chromatin and pale-colored abundant cytoplasm containing basophilic granules.The neoplastic lymphocytes showed an increased number of mitotic figures and Ki-67-positive cells (MC: 70/10 HPFs and MIB-1 index: 49%). Binucleated cells were frequently found (Fig. 1). Inflammatory infiltrates of plasma cells and eosinophils was observed. The animal was treated with a combination of prednisolone (1–2 mg/kg), vincristine (0.025 mg/kg, administered intravenously) or cyclophosphamide (10 mg/kg, administered subcutaneously) approximately a week apart. Although lethargy and anorexia were seen for 3 days after the introduction of chemotherapy, the animal's general condition and hematology remained within normal limits. The size of the tumor slightly reduced after the first round of vincristine chemotherapy; however, the tumor continued to grow despite treatment. The animal died 6 weeks after the initial treatment. No necropsy was conducted.

Case 2. A 10-year-old intact female prairie dog was presented with one month history of swelling of the right cheek and the sudden onset of anorexia and lethargy. An oral mass was found in the gingiva and oral mucosa during a physical examination. Microscopically, moderate to large, pleomorphic neoplastic lymphocytes had predominantly infiltrated the edematous lamina propria, including the resection margins (Fig. 2). These neoplastic lymphocytes exhibited polygonal or horseshoe-shaped atypical nuclei with dense chromatin and abundant pale cytoplasm with or without basophilic granules. The neoplastic lymphocytes were accompanied by binucleated cells and inflammatory cells. The MC (70/10 HPFs) and MIB-1 index (57%) were remarkably high. The animal died on the day of the biopsy examination. No necropsy was conducted.

Case 3. An intact female prairie dog that had been kept in a breeding colony was found dead and presented for necropsy. Clinical data, including the animal's age, were not available. The animal died suddenly without any specific clinical symptoms. The post-mortem findings included pulmonary edema and marked splenomegaly and hepatomegaly. Significant hemorrhaging and multi-focal necrosis were seen in the congested liver. The markedly enlarged mesenteric lymph nodes and adjacent intestines, including the distal ileum, cecum and colon, were pale and edematous. A microscopic examination of the mesenteric lymph nodes

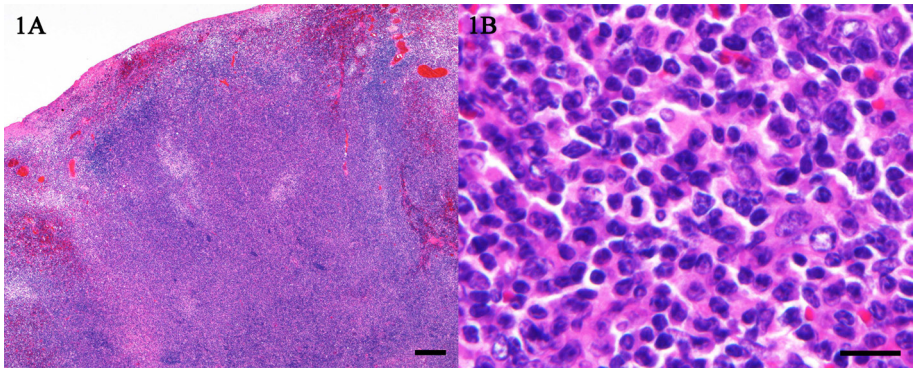

Fig. 1. Case No. 1, cutaneous ALTCL. (A) Lower magnification showed the ulcerated epithelium, missing cutaneous adnexal structures and tumor cells filled in the dermis. HE. Bar, 500 μm. (B) Higher magnification showed coarsely clumped chromatin and binucleation of tumor cells. HE. Bar, 40 μm.

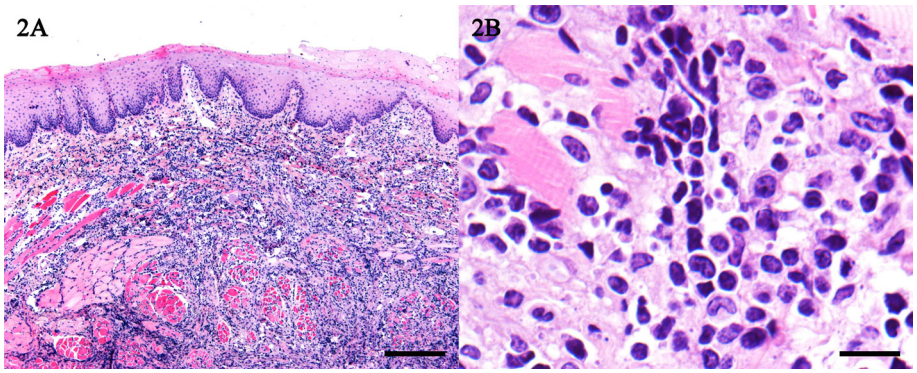

Fig. 2. Case No. 2, oral ALTCL. (A) Diffuse proliferation of tumor cells in the lamina propria. HE. Bar, 500 μm. (B) Higher magnification showed tumor cells with horseshoe-shaped atypical nuclei. HE. Bar, 40 μm.

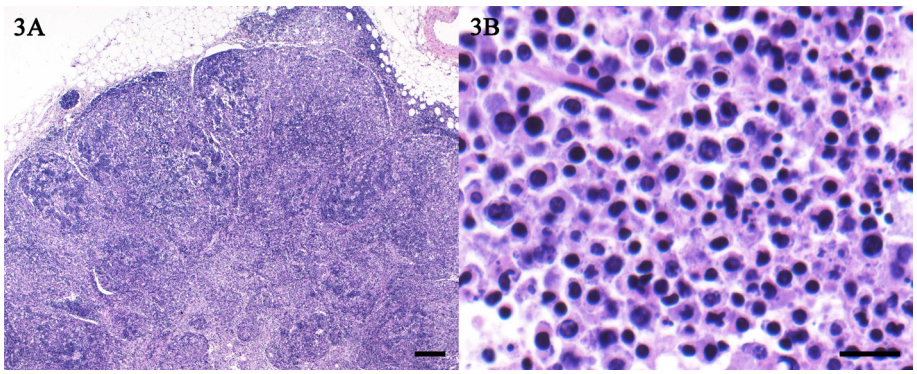

Fig. 3. Case No. 3, nodal ALTCL. (A) Diffusely proliferating tumor cells compress follicular structures of the lymph node. HE. Bar, 500 μm. (B) Tumor cells consist of a large amount of cytoplasm and pleomorphic nuclei. HE. Bar, 40 μm.

revealed distortion of the normal lymphoid architecture (Fig. 3), together with compressed germinal centers surrounded by large round to ovoid pleomorphic neoplastic lymphocytes. The paracortical region was densely filled with neoplastic lymphocytes. The neoplastic lymphocytes contained a large amount of cytoplasm with eosinophilic granules and pleomorphic nuclei, which contained dense and coarsely clumped chromatin. The neoplastic lymphocytes frequently infiltrated dilated lymphatic vessels. Significantly high mitotic activity (MC: 68/10 HPFs and MIB-1 index: 50%) and binucleated cells were often found in the tumor

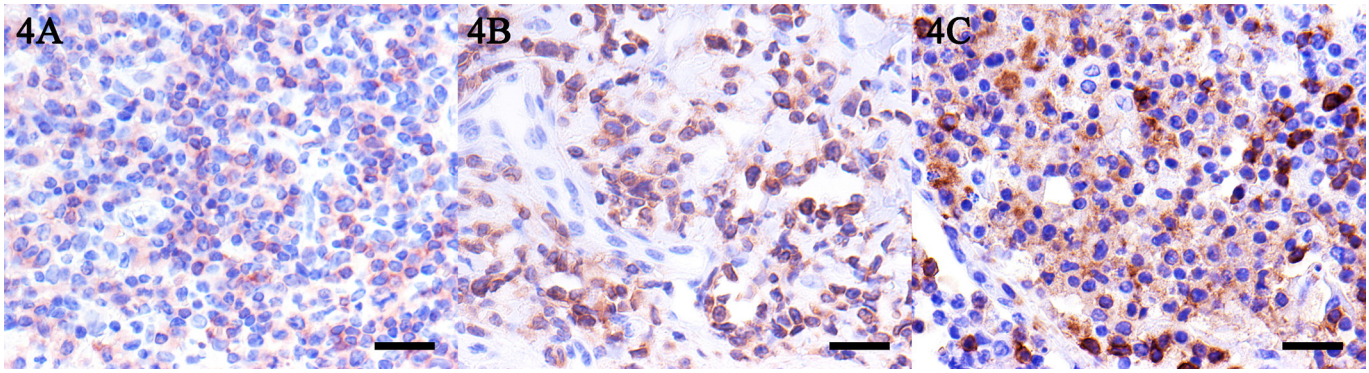

Fig. 4. Immunohistochemical staining of CD3 reveals cytoplasmic or membranous staining of varying degrees in (A) Case No. 1, (B) Case No. 2 and (C) Case No. 3. IHC. Bar, 40 μm.

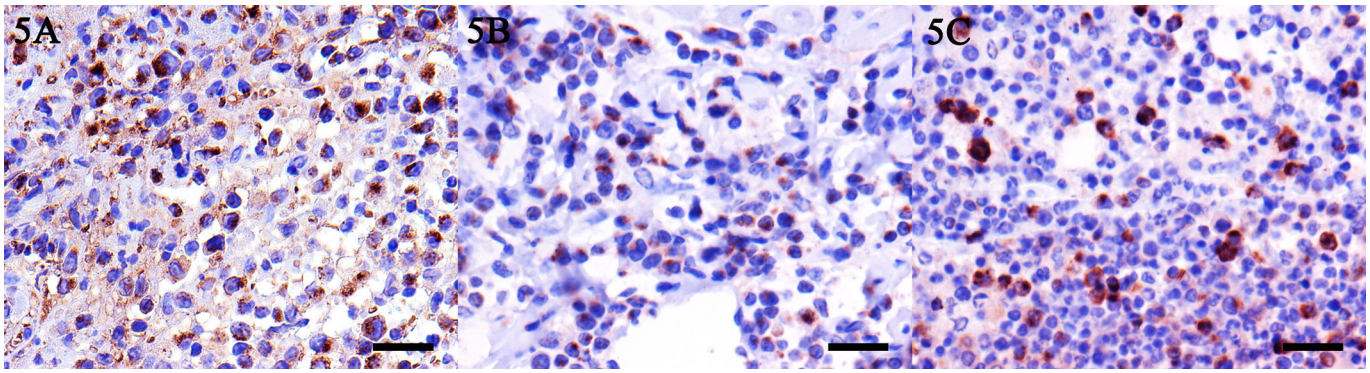

Fig. 5. Immunohistochemical staining of granzyme B shows a cytoplasmic granular pattern in (A) Case No. 1, (B) Case No. 2 and (C) Case No. 3. IHC. Bar, 40 μm.

Table 3. Histopathology and immunohistochemistry of the neoplastic lymphocytes

Case No.	Nuclear morphology	Nuclear size relative to RBC	Nuclear atypia	Cytoplasm	MIB-1 index	CD3	CD20	CD79	CD56	Iba-1	Granzyme B
1	Ovoid, polygonal	>3	High	Abundant	49	IC	-	-	-	-	GC
2	Polygonal, horseshoe-shaped	>3	Moderate	Abundant	57	M/IC	-	-	-	-	GC
3	Round, ovoid	>3	High	Abundant	50	M/IC	-	-	-	-	GC
Control						M (T cells)	M (B cells)	M (B cells)	M (NK cells)	M (Mϕ cells)	GC (Intraepithelial lymphocytes)

IC, intracytoplasmic; M, membranous; GC, granular cytoplasmic; (−), negative; RBC, red blood cells; the staining patterns were compared with those of the internal positive control.

nests. Neoplastic lymphocytes were also observed in the large intestines, small intestines, liver, lungs, spleen, kidneys and adrenal glands. The marked infiltration of neoplastic lymphocytes was also noted in the cecal lamina propria and hepatic portal region. No epitheliotropism or angiotropism of the neoplastic cells was evident in the tumor nests. The neoplastic lesions were often accompanied by eosinophil and plasma cell infiltration.

Immunohistochemically, intracytoplasmic CD3 expression was detected in the neoplastic cells in all cases, although it varied in intensity. The neoplastic lymphocytes in case 1 displayed subtle to moderate intracytoplasmic CD3 expression, while intracytoplasmic and distinctive membranous staining patterns were observed in cases 2 and 3 (Fig. 4). In all cases, the neoplastic lymphocytes demonstrated a granular cytoplasmic granzyme B-staining pattern (Fig. 5). Double immunofluorescent staining showed the colocalization of CD3 and granzyme B in all cases. The MIB-1 indices of 49, 57 and 50 were seen in cases 1, 2 and 3, respectively. The neoplastic cells were negative for CD79α, CD20, CD56 and Iba-1. The morphological and immunohistochemical features of the three cases are summarized in Table 3.

According to their morphological and immunohistochemical features, all three black-tailed prairie dogs were diagnosed with ALTCL. In case 1, it was not possible to evaluate the epitheliotropism of the neoplastic cells due to marked ulceration. The animal in case 2 was diagnosed with oral ALTCL. ALTCL of the oral mucosa is rarely reported in humans [5, 7]. In case 3, nodal ALTCL

of the mesenteric lymph nodes and systemic dissemination were observed. The World Health Organization classification of the tumors affecting domestic animals describes ALTCL as possessing neoplastic lymphocytes with peripheralized chromatin, irregular parachromatin clearing, prominent nucleoli and abundant cytoplasm [10]. The morphological features of cases 3 (nodal) and 1 (cutaneous) were similar to those described in a previous study [9].

CD3 expression was observed in all of the prairie dogs although it exhibited varying degrees of intensity and staining patterns, whereas it is often absent in canine and feline ALTCL [9]. In contrast to the usual membranous CD3 staining pattern, the ALTCL cells in the present study displayed a weak to moderate intracytoplasmic CD3-staining pattern with or without membranous expression. Histiocytic sarcoma, the primary differential diagnosis of ALTCL [9], was ruled out based on the lack of Iba-1 expression. An anaplastic variant of diffuse large B-cell lymphoma was ruled out due to the absence of CD20 and CD79α expression.

The cytoplasmic granular expression of granzyme B is indicative of the cytotoxic T cell phenotype [9]. Granzyme B is a serine protease that is frequently found in cytoplasmic granules within lymphocytes with cytotoxic potential. Neoplastic ALTCL cells are often positive for granzyme B in humans and domestic animals [1, 9]. However, granzyme B expression cannot be used to predict the prognosis of human ALTCL cases [1]. In the present study, the consistent expression of granzyme B was demonstrated in all cases, indicating that the neoplastic cells were of cytotoxic T cell-origin. In addition, all of the current cases exhibited high MIB-1 indices (mean MIB-1 index: 52%), which might be associated with malignant behavior.

In human cases, the cutaneous type of ALTCL has been reported to have a better prognosis than the nodal type [2, 4]. One human case of oral ALTCL underwent spontaneous regression [5]. In contrast, ALTCL with peripheral blood involvement often has a poor prognosis in humans [6]. In the present study, the neoplastic lymphocytes exhibited high proliferative rates in all cases. In case 1, the animal died after six weeks of chemotherapy. In cases 2 and 3, the animals died on the day of the biopsy examination and suddenly without specific symptoms, respectively. These findings suggest that in black-tailed prairie dogs, ALTCL is highly aggressive and has a poor prognosis, as has been described for other domestic animals [9].

In conclusion, the present study described cases of ALTCL in black-tailed prairie dogs. The tumors were of cytotoxic T cell origin and exhibited consistent granzyme B expression. The poor response to chemotherapy, rapid progression and high proliferative rate seen in these cases suggest that ALTCL has a poor prognosis in black-tailed prairie dogs.

CONFLICT OF INTEREST. None of the authors have any conflicts of interest to declare.

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