T-Cell Lymphoma in a Wild Okinawa Rail (Gallirallus okinawae)

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ABSTRACT. The Okinawa rail (Gallirallus okinawae) is an endangered species that inhabits the northern part of Okinawa Main Island in southern Japan. A wild Okinawa rail was rescued from a road in Kunigami Village in Okinawa in October 2009. The bird subsequently died and underwent necropsy. Tumors were found in the liver, spleen and part of the small intestine. Microscopically, lymphoid neoplasm was confirmed in these tissues. The tumor cells were mainly positive for CD3 and CD8 by immunohistochemistry. No Marek’s disease virus genes were detected by PCR of a liver tumor. This is the first report of T-cell lymphoma in the Okinawa rail.

KEY WORDS: immunohistochemistry, lymphoma, Okinawa rail, polymerase chain reaction.

NOTE: Avian Pathology

The Okinawa rail (Gallirallus okinawae) is a member of the family Rallidae and is endemic to the northern part of Okinawa Main Island in southern Japan. It is classified as an endangered species according to the International Union for Conservation of Nature (IUCN) red list categories [1]. The population size of the Okinawa rail was estimated to be 820–1,300 in 2006 [13]. The population in 2006 had decreased to approximately 40% of that estimated in 1985 (1,500–2,000 birds) [13].

Lymphoid neoplasm in domestic poultry can be associated with viral oncogenesis involving Marek’s disease virus (MDV), lymphoid leukemia virus (LLV) and reticuloendotheliosis virus (REV) [24]. In wild birds, lymphoid leukemia-like lesions are the most frequent tumor reported [6]. However, the connection between neoplasia and the presence of a virus in most birds has not been demonstrated [6].

Lymphoma has not previously been reported in Okinawa rail. This is the first report of lymphoma in an Okinawa rail. A conservation plan for Okinawa rails is currently being implemented in Japan, and this case raises concerns regarding possible impacts on the health of this endangered species.

On 27 October 2009, an adult female Okinawa rail (ID No. 1194A) was rescued from a road in Kunigami Village on Okinawa Main Island, and it was suspected that it had been hit a car. The bird was emaciated, and its body weight was 306 g (average female weight = 413.5 g; n=20 [14]). It showed head tremor and left periorbital swelling. It had no bone fractures or traumas. A blood test was performed using a VetScan VS2 Clinical Biochemistry Analyzer (Abaxis®, Union City, CA, U.S.A.) and heparinized whole blood from the caudal tibial vein. The bird showed hypoglycemia (1.3 mM), high aspartate amino transferase (AST; 1028 U/l) and high creatine kinase (CK; 5630 U/l). Despite treatment with hemostat, antibiotics, steroids and glucose, the bird died on 28 October 2009.

Necropsy revealed Milky white, soft tumors in the liver, spleen and part of the small intestine, which was located 1 cm back from the Meckel’s diverticulum. The liver was enlarged with round edges and had diffuse, multifocal, white raised foci on the surface (Fig. 1A), as well as in cross section. These foci infiltrated into the adjacent tissues with indistinct boundaries. The spleen was also enlarged to about three times larger than the normal spleen (Fig. 1B). The cut surface of the spleen was milky white in color and soft. Part of the mucus membrane of the small intestine located near the Meckel’s diverticulum was thickened and was milky white in color. The cranial lobes of the kidneys were slightly swollen and congested. The thymus glands were not enlarged and appeared to be normal. There was no hemorrhage or other injury of the head and brain. Left peri orbital swelling was not seen at necropsy; therefore, the swelling was not related to the tumors. There was a hemorrhage on the left deep pectoral muscle. The heart, lung, ovary, skin, bursa of Fabricius, nerves, thyroid glands and proventriculus appeared to be normal.

The liver, spleen, heart, lung, kidney, brain, ovary, skeletal muscle, skin, thymus gland, thyroid gland, proventriculus, bursa of Fabricius, brachial plexuses, sciatic nerves and small intestine were fixed in 10% neutral buffered formalin, routinely processed for histology and stained with hematoxylin and eosin (HE). Histopathologic examination revealed small- to large-sized neoplastic lymphoblast cells infiltrating tumors of the liver (Fig. 1C), spleen, kidney, thymus and...
part of the small intestine. Large size neoplastic cells had deep eosinophilic cytoplasm and dense nuclear chromatin. Other small size neoplastic cells had hyperchromatic nuclei. Neoplastic cells effaced the liver structure, and few hepatic cells remained. Large numbers of neoplastic cells disturbed the normal spleen structures of the splenic trabeculae and

Fig. 1. (A) Enlarged liver with round edges showing diffuse, multifocal, white raised foci on the surface. Scale bar=1 cm. (B) The spleen was enlarged to about three times the size of a normal spleen. Scale bar=1 cm. (C) Small to large-sized lymphocytes infiltrating the liver. There were cells enriched with cytoplasm, cells with dense chromatin and small cells with hyperchromatic nuclei. Mitotic figures were seen in the liver (arrows). HE stain. Scale bar=20 μm. (D) CD3 was strongly positive in the 1194A liver tumors. Scale bar=20 μm. (E) CD8α was positive in small to large neoplastic cells in the liver tumor. Scale bar=50 μm. (F) A small number of scattered BLA.36-positive cells was also detected within the small neoplastic cells in the liver tumor. Scale bar=20 μm.
germinal centers. The tumor proliferates in the kidney were located in the interstitial tissue. Pleomorphic neoplastic lymphoblasts also disturbed the normal thymus structure, and few Hassall’s bodies remained. Neoplastic lymphoblasts infiltrated and expanded the tunica serosa, tunica muscularis and submucosa of part of the small intestine. Mitotic figures were present in the liver, kidney, thymus and spleen lesions with up to almost 3 per high power field. Microscopic evaluations of other tissues (heart, lung, brain, ovary, muscle, skin, thyroid gland, proventriculus, bursa of Fabricius and nerves) were unremarkable.

Immunohistochemistry can help to discriminate between T-cell tumor and B-cell tumor in poultry [9, 18], and we therefore used immunohistochemistry to investigate the etiology of the Okinawa rail’s tumor. Immunohistochemistry was performed on the liver, spleen, thymus and small intestine (Table 1). Paraffin-embedded organs were cut into 3-µm sections and heated in an autoclave for 20 min at 121°C with a target retrieval solution (S2031, Dako, Glostrup, Denmark). An EnVision™ System-HRP with DAB (K1390, Dako, Kyoto, Japan) was used as the detection system, according to the manufacturer’s instructions. T-cell and B-cell tumors were distinguished using commercially available antibodies. Polyclonal rabbit anti-human CD3 (N1580, Dako, Carpinteria, CA, U.S.A.) at no dilution, mouse anti-chicken CD4 (733042, Beckman Coulter®, Fullerton, CA, U.S.A.) at 1:50 dilution and mouse anti-chicken CD8α (733139, Beckman Coulter®, Fullerton, CA, U.S.A.) at 1:100 dilution were used as T-cell markers. Monoclonal mouse anti-human BLA.36 (BioGenex, San Ramon, CA, U.S.A.) at 1:50 dilution was used as a B-cell marker. The reaction time for CD3 and BLA.36 was 1 hr in a humid chamber at room temperature. The reaction time for CD4 and CD8α was 18 hr in a humid chamber at room temperature. The effectiveness of these antibodies for the Okinawa rail was confirmed using normal spleen and bursa of Fabricius tissues from other Okinawa rails (Table 1). A skin tumor from a Japanese bantam with Marek’s disease and a liver tumor from another bantam with B-cell lymphoma were also used as controls (Table 1). The results confirmed that CD3, CD8α and BLA.36 showed cross-reactivity in Okinawa rail lymphocytes. CD4 did not react with any tissue sections.

CD3 was strongly positive in relatively small neoplastic cells in the liver (Fig. 1D), spleen, thymus and intestine tumors. CD3-positive cells were distributed over the whole sections of these tumors. CD8α was positive in many small to large neoplastic cells in the liver (Fig. 1E) and spleen. These pathologic findings suggested that the lymphoma was associated with T-cell lymphocytes. A small number of scattered BLA.36-positive cells was also detected in small neoplastic cells in liver (Fig. 1F), spleen and thymus lesions. Although the distribution of BLA.36-positive cells was limited, they were not found in any specific location.

In the case of MDV, the majority of lymphoid cells of the tumor are T lymphocytes, with only 3 to 10% of B lymphocytes present [18]. MDV tumor cells are pleomorphic types of cellular infiltration composed of a mixed population of small to large lymphocytes [3]; therefore, PCR was performed to detect MDV. DNA was extracted from the fresh liver and spleen tumors and from feather tips using an EZ1® DNA Tissue Kit (Qiagen, Tokyo, Japan), according to the manufacturer’s instructions. Feather tips were sampled from both wings, as well as from caudal, abdominal, pectoral and dorsal regions. The primer sets for MDV were SORF2F, TGGATTTGGTACATGAAAGGA, and SORF2R, TCCATCTTAAACAGGTGTGG [11]. The expected amplified DNA band size was 231 bp. Amplification was carried out using AccuPrime™ Taq DNA Polymerase (Invitrogen, Paisley, U.K.). The reaction conditions were 94°C for 2 min for initial denaturation, followed by 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 68°C for 1 min. Samples were run on 2% agarose gels to confirm the presence of PCR products. DNA extracted from paraffin-embedded skin tumor sections from a Japanese bantam with Marek’s disease (C-2; Table 1) was used as a positive control. MDV DNA was detected in C-2 by PCR, but not in 1194A.

The rescued Okinawa rail was suspected to have been hit by a car because it was lying on a road and had head tremor, peri-orbital swelling and high values of AST and CK. It also showed a hemorrhage on the left deep pectoral muscle in necropsy. These results indicated damage due to being hit by a car. On the other hand, it showed weight loss, hypoglycemia and high values of AST. These results indicated damage due to malignant tumors. Thus, the direct cause of this Okinawa rail’s death was unknown.

Small neoplastic cells in tumors of the liver, spleen, thymus and intestine were positive for CD3. In addition, many small to large neoplastic lymphoblasts in tumors of the liver and spleen were positive for CD8α. We therefore concluded that the tumor in this rail was a T-cell lymphoma.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Age class</th>
<th>Cause of death</th>
<th>Target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1194A</td>
<td>Okinawa rail</td>
<td>A</td>
<td>Lymphoma/car accident</td>
<td>Tumors in liver, spleen, thymus and/or intestine</td>
</tr>
<tr>
<td>1023A</td>
<td>Okinawa rail</td>
<td>J</td>
<td>Unknown</td>
<td>Normal spleen</td>
</tr>
<tr>
<td>1247A</td>
<td>Okinawa rail</td>
<td>A</td>
<td>Car accident/predation</td>
<td>Normal bursa of Fabricius</td>
</tr>
<tr>
<td>A-2</td>
<td>Bantam</td>
<td>A</td>
<td>B-cell lymphoma</td>
<td>Liver tumor</td>
</tr>
<tr>
<td>C-2</td>
<td>Bantam</td>
<td>A</td>
<td>Marek’s disease</td>
<td>Skin tumor</td>
</tr>
</tbody>
</table>

a) A = adult. b) J = juvenile.
A possible reason for the small number of BLA.36-positive neoplastic cells also seen in the tumors is the staining ability of BLA.36. BLA. 36 has been reported to rarely label T-cell tumors [4]. Another possibility is that CD3-positive cells coexpress BLA. 36. The phenomena that CD3-positive cells coexpress CD79, a B-cell marker, has been reported in some lymphoblastic lymphoma in humans [16]. Also, canine lymphoma cases with T- and B-cell marker coexpression represent aggressive T-cell lymphoma/leukemias [22].

The possible reason for CD4 not reacting with any tissue sections is failure to retrieve antigenicity from paraffin-embedded sections. Basically, anti-chicken CD4 and CD8α can be used on acetone-fixed, frozen tissue sections. The availability of these antibodies for paraffin-embedded sections was unknown. In human cases, paraffin-embedded sections can be used for CD4 and CD8 with the appropriate antigen retrieval method [12]. Therefore, the antigen retrieval methods require further consideration.

In the case of free-ranging wild birds, multicentric lymphomas have been reported to be associated with MDV in a white-fronted goose (Anser albifrons), LLV in a European Starling (Sturnus vulgaris) and REV in a wild turkey (Meleagris gallopavo) [8, 10, 21]. LLV was excluded in the present case because the tumors were T-cell lymphomas. From the gross lesions, morphology of the neoplastic cells and immunohistochemistry of T-cells, we suspected Marek’s disease; however, no MDV genes were detectable by PCR. Furthermore, CD8α-positive neoplastic lymphoblasts were found in tumors. MD is a naturally occurring lymphoma of mostly CD4+CD8- T cells [19]. These PCR and immunohistochemical results suggest that this case is not associated with MD. In the case of REV, T and/or B cells have been observed in tumor lesions [15]. REV infection has been associated with bursal and nonbursal lymphomas [23]. Nonbursal lymphomas are believed to be of T-cell origin [25]. Furthermore, natural infection with REV results in an increase in CD8+ lymphocytes and a decrease in CD4+ lymphocytes [5]. Further investigation of REV is required.

Although lymphoid tumors have been reported from many avian species and described as Marek’s disease or lymphoid leukemia, the causative viruses have been rarely isolated [6, 17]. While lymphoid neoplasm can be associated with viral oncogenesis (e.g., MDV, LLV and REV), there has been a report of lymphoid neoplasm in parrots with non-viral etiology [2]. Investigation of non-viral etiology is also required.

This is the first report of lymphoma in an Okinawa rail. Because the Okinawa rail is an endangered species in Japan, further investigations into the etiology of the lymphoma and observations of the incidence of lymphoma within the Okinawa rail population are needed to promote their effective in situ and ex situ conservation.

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REFERENCES

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