NOTE *Pathology*

Combined Hepatocellular-Cholangiocarcinoma in a Yellow-Headed Amazon (*Amazona oratrix*)

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ABSTRACT. A 9-year-old male Yellow-headed Amazon (*Amazona oratrix*) with a history of anorexia and vomiting died of a liver tumor. The tumor consisted of neoplastic cells with hepatocellular and cholangiocellular differentiations and their intermingled areas. Neoplastic hepatocytes showed islands or trabecular growth with vacuolated eosinophilic cytoplasm. Cells showing biliary differentiation formed ducts or tubules lined by cytokeratin AE1/AE3-positive epithelia, accompanied by desmoplasia consisting of myofibroblasts reacting to α-smooth muscle actin and desmin. The tumor was diagnosed as a combined hepatocellular-cholangiocarcinoma, which is very rare in the avian.
KEY WORDS: combined hepatocellular-cholangiocarcinoma, immunohistochemistry, liver, Yellow-headed Amazon.

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Hepatocellular carcinomas and cholangiocarcinomas are common liver malignant tumors in humans and animals [2]. On the contrary, combined hepatocellular-cholangiocarcinoma (CHCC) is rare in all animal species [19]. According to the World Health Organization histological classification of tumors of the domestic animals, CHCC is also termed hepatocholangiocarcinoma, which shares clear histopathological features of both hepatocellular carcinoma and cholangiocarcinoma [8]; differentiated cells toward hepatocytes and bile duct epithelia coexist in the same tumor [8]. In the avian, CHCC has been reported in four cases of domestic ducks [14], one case of a layer chicken [5] and one case of a lesser flamingo [19]. Because of the rarity, here we report histopathological and immunohistochemical characteristics of CHCC encountered in a Yellow-headed Amazon, which is the first case reported in this species of the avian.

A 9-year-old male Yellow-headed Amazon (*Amazona oratrix*), 700 g in body weight, kept as a pet was brought to a private animal hospital following anorexia, vomiting and anemia. Barium contrast X ray examination showed intestinal compression, and an intra-abdominal mass was detected by echographic examination. Several days later, the bird died. At necropsy, an enlarged liver with irregular/nodular surface and yellowish transparent ascitic fluid were seen; heart, lung, liver, kidney, spleen, adrenal glands, pancreas, testes and intestines were fixed in 10% neutral buffered formalin for histopathological examination. Paraffin-embedded tissues were cut at 4 μm in thickness. Beside hematoxylin and eosin (HE) stain, deparaffinized sections were stained with Alcian blue at pH 1.0 and 2.5, azan-Mallory stain and Prussian blue stain. Frozen sections from formalin-fixed tissue, cut at 10 μm in thickness, were stained with oil red O stain. Sections were also immunolabeled with mouse monoclonal antibodies for desmin (1:200; Dako, Carpinteria, CA, U.S.A.), alpha-smooth muscle actin (α-SMA; 1:500; Dako), cytokeratin 19 (CK19; 1:100; Novocastra Laboratories Ltd., Newcastle, U.K.), cytokeratin AE1/AE3 (1:1, 000; Dako) and proliferating cell nuclear antigen (PCNA; 1:500; Dako). For α-SMA, no pre-treatment was done. Sections for CK19 were treated with trypsin at 37°C for 20 min. For the other markers, sections were pre-treated with heat (microwave in citrate buffer for 20 min). All sections were then treated with 3% H_2O_2 in phosphate buffered saline (PBS) to quench endogenous peroxidase activity and then with 5% skimmed milk to inhibit non-specific reactions. The sections were incubated with a primary antibody overnight at 4°C and then reacted with peroxidase-conjugated secondary antibody (Histofine Simple Stain, Nichirei, Tokyo, Japan). Positive reactions were visualized with 3, 3′-diaminobenzidine (DAB Substrate Kit, Vector Laboratories Inc., Burlingame, CA, U.S.A.). Sections incubated with non-immunized serum and appropriate sections specific for each primary antibody were used as negative and positive controls, respectively. Sections were counterstained lightly with hematoxylin. In addition to identify the co-expressions of antigens recognized by AE1/AE3 and PCNA, the modified double immunohistochemistry was performed according to the method described by Hasui *et al*. [7]. First, sections were immune-labeled with AE1/AE3 antibody and visualized red by the Fuchsin substrate-chromogen system (Dako). Second, sections used for AE1/AE3 were reacted with PCNA antibody. The positive reactions at the second labeling were visualized brown

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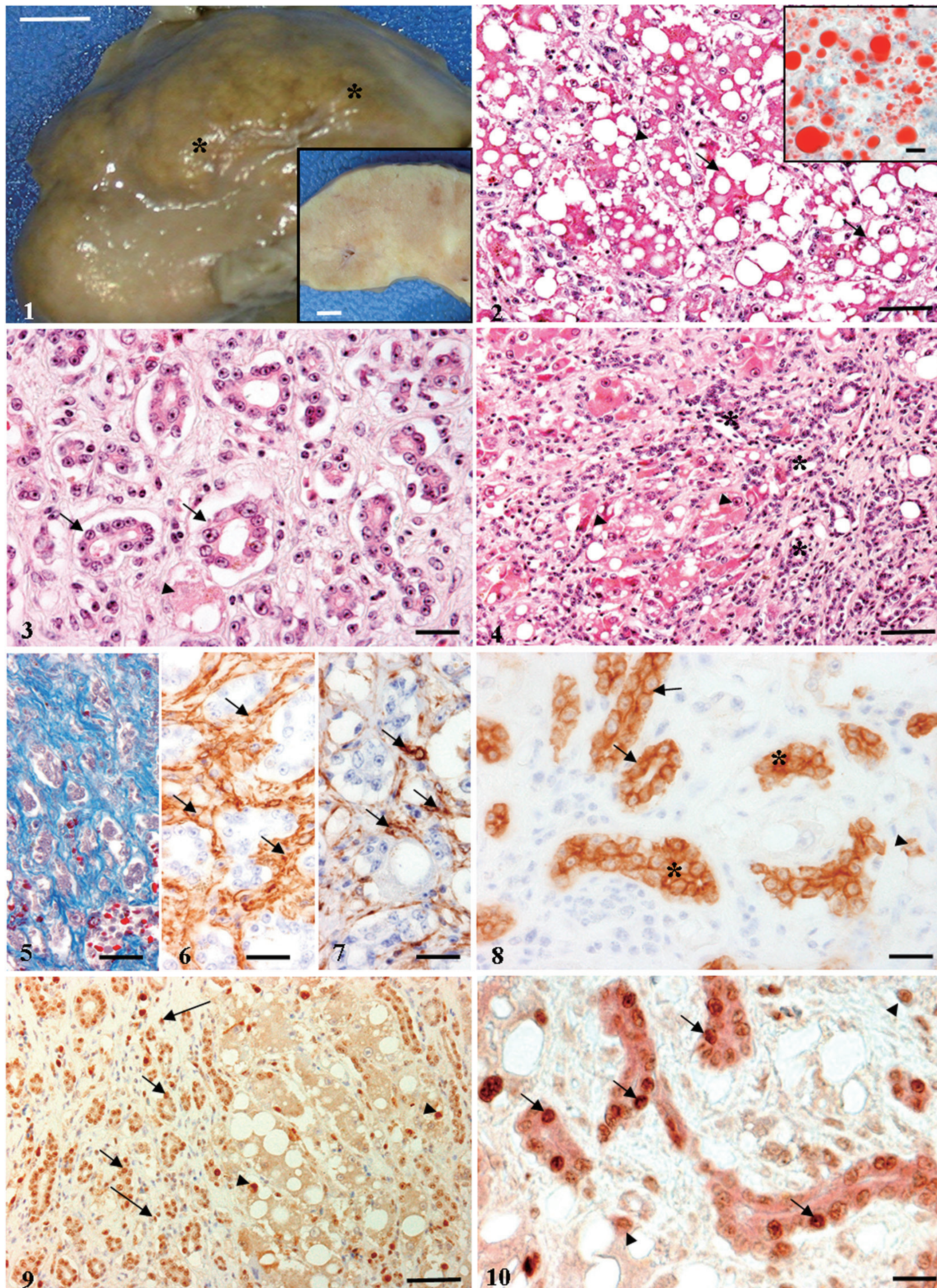

Fig. 1. Gross finding. The liver shows pale brown color; and has irregular surface and nodular masses of 1–3 cm in diameter (asterisks). Inset shows the cut surface after formalin fixation. Bar=1 cm.

Fig. 2. Area consisting of islands of cells with hepatocellular differentiation with vacuolated eosinophilic cytoplasm (arrows) and areas with trabecular growth (arrowhead). HE stain. Bar=50 μm. Inset shows cytoplasmic vacuoles stained red by oil red O stain. Bar=20 μm.

Fig. 3. Biliary differentiation area consisting of ducts or tubules lined by monolayered columnar epithelial cells (arrows) and abundant collagen (desmoplastic stroma). HE stain. Bar=20 μm. An arrowhead indicates neoplastic hepatocytes.

Fig. 4. Intermediate differentiation area composed of an admixture of hepatocellular (arrowheads) and cholangiocellular components (asterisks). HE stain. Bar=50 μm.

with DAB as mentioned above. The percentage of PCNA-positive cells was counted in hepatocytes, biliary epithelial cells and interstitial cells by counting each 1,000 cells at a high power magnification.

Grossly, the liver was moderately enlarged and had some nodular lesions of 1–3 cm in diameter. Formalin-fixed liver was pale brown in color (Fig. 1), and the cut surface was moderately firm and uniformly whitish (Fig. 1, inset). No gross lesions were found in other organs, such as lungs, heart, kidneys, spleen, adrenal glands, pancreas and alimentary tracts. Histopathologically, the affected liver was composed of neoplastic hepatocytes with vacuolated eosinophilic cytoplasm arranged in islands or trabecular growth (Fig. 2); there were no normal structures, such as hepatic cords, Glissons's sheath and central veins. Areas of biliary differentiation consisting of neoplastic ducts and tubules were also present in the affected liver; these tubules were lined by monolayered columnar epithelial cells, including sporadic clusters of neoplastic hepatocytes (Fig. 3). In addition, there were areas composed of an admixture of hepatocellular and cholangiocellular components, as well as their intermediate differentiation (Fig. 4). In areas of biliary differentiation, interestingly, abundant desmoplastic stroma (Fig. 3), stained blue by azan-Mallory stain, was present among neoplastic bile ducts and tubules (Fig. 5); the stroma showed a faint reaction to Alcian blue; desmoplastic stroma-consisting cells reacted strongly to α-SMA (Fig. 6) and desmin (Fig. 7), indicating that they are myofibroblastic cells. In areas of hepatocellular differentiation, the cells had round to polygonal nuclei with one or two prominent nucleoli; the cells contained abundant cytoplasm with hemosiderin granules, demonstrable with Prussian blue stain, and their cytoplasmic vacuoles were stained red by oil red O stain (Fig. 2, inset). Neoplastic cells with biliary differentiation were stained positively with cytokeratin AE1/AE3 antibody; the positive cells often formed ducts and tubules, and there were also clusters consisting of several cells without cavity and a single cell (Fig. 8). CK19 immunohistochemistry failed to give a positive reaction; however, the rat liver used as the control gave a positive reaction. Many cells in the areas of hepatocellular differentiation and biliary differentiation showed the proliferating activity, demonstrated by the PCNA immunolabeling (Fig. 9). The mitotic index was higher in biliary differentiation areas, accounting for approximately 60% of total biliary epithelial cells, as compared with hepatocellular differentiation cells (approximately 40%) and interstitial cells in the desmoplastic stroma (approximately 30%). Double immunolabeling indicated that the AE1/AE3-positive biliary differentiation cells had the PCNA-positive proliferating activity (Fig. 10).

In other organs examined, no significant changes, such as neoplastic lesions, were detected, except for focal necrosis in myocardia.

According to the World Health Organization histological classification of tumors of domestic animals, CHCC should have clear cellular differentiations resembling both hepatocytes and biliary epithelia [8]. In addition, an interface of these 2 components including an intermingle differentiation intimately with each other should also be appreciated [22]. Hepatocellular carcinoma with pseudo-glandular and trabecular growth patterns may make CHCC diagnosis difficult [12]. Cytokeratin AE1/AE3 is considered to be the most useful marker for indistinguishing cholangiocellular areas in CHCC from hepatocellular carcinomas; neoplastic cells towards hepatocyte differentiation did not react to AE1/AE3, whereas neoplastic cells showing biliary differentiation reacted clearly to the antibody [6, 21]. In the present case, there were cells reacting to AE1/AE3 which formed ducts and tubules or appeared as clusters without lumina, denoting cholangiocellular origin; in some areas, these cells were predominant. Cholangiocarcinoma may be characterized by severe proliferation of stromal cells [9]. In the present CHCC, cholangiocellular areas consisted of highly desmoplastic stroma which consisted of myofibroblasts positive for α-SMA and desmin and abundant collagen fibers. We tried immunohistochemistry with CK19 antibody, a helpful marker for the differential diagnosis of hepatocytes and bile ducts in mammals [21], but the antibody was not found to react with avian cytokeratin. Antibodies for albumin and CK8/18 may be used for demonstration of hepatocellular component with trabecular growth pattern in mammals [20]. However, there have been no antibodies useful for avian hepatocytes. The presence of PCNA-positive hepatocytes in the present tumor indicated neoplastic proliferation of cells showing hepatocellular differentiation.

Based on the invasive growth, the structural and nuclear atypia with high mitotic index and the presence of areas of hepatocellular and cholangiocellular differentiations, the present case was diagnosed as a CHCC. The histogenesis

Fig. 5. Desmoplastic stroma in the cholangiocellular differentiation area; the stroma is stained blue by the azan-Mallory stain among ducts or tubules formed by neoplastic cholangiocytes. Azan-Mallory stain. Bar=50 μm.

Fig. 6. The desmoplastic stroma in the cholangiocellular differentiation area consisting of α-smooth muscle actin-positive myofibroblasts (arrows). Immunohistochemistry, counterstained with hematoxylin. Bar=20 μm.

Fig. 7. The desmoplastic stroma in the cholangiocellular differentiation area consisting of desmin-positive myofibroblasts (arrows). Immunohistochemistry, counterstained with hematoxylin. Bar=20 μm.

Fig. 8. Cells forming ducts or tubules (arrows) in the cholangiocellular differentiation area react to cytokeratin AE1/AE3. Some AE1/AE3-positive cells are present without forming cavity (asterisks) or as a single cell (arrowhead). Immunohistochemistry, counterstained with hematoxylin. Bar=20 μm.

Fig. 9. Neoplastic cells showing the proliferating activity, demonstrated by the PCNA immunolabeling in the cholangiocellular differentiation area (short arrows) and in hepatocellular differentiation area (arrowheads). Some interstitial cells (long arrows) show a PCNA positivity. Immunohistochemistry, counterstained with hematoxylin. Bar=50 μm.

Fig. 10. PCNA-positive, proliferating biliary epithelial cells react to AE1/AE3 (arrows). Double immunolabeling for AE1/AE3 (red in cytoplasm) and PCNA (brown in nuclei). Cells reacting to PCNA, but negative to AE1/AE3 are neoplastic hepatocytes (arrowheads). Bar=20 μm.

of CHCC is still debated. Three possibilities have been proposed: 1. a collision tumor of hepatocellular and cholangiocellular carcinomas which develops simultaneously and independently: 2. a cancer that first developed either from the hepatocyte or bile duct epithelium and subsequently develops into other component (carcinoma in carcinoma): 3. a cancer which develops from a progenitor cell with capacity to differentiate towards both hepatocytes and cholangiocytes [10, 11]. Recently, it has been considered that human CHCC may derive from hepatic progenitor cells, and depending on micro-environmental conditions, the cells can differentiate towards hepatocyte or cholangiocyte differentiation [16, 17]. Hepatic progenitor cells can be identified by co-expression of hepatocytic and cholangiocytic lineage markers (HepPar1 and CK19) as well as hematopoietic stem cell markers, such as c-Kit and CD34 [1, 18]. Unfortunately, these antibodies are not available for avian tissues.

As for the etiology, it has been considered that some CHCC and hepatocellular carcinoma in humans may be due to hepatitis C and B virus infection [13]. In veterinary medicine, hepatic malignant neoplasms can arise after hepatic injury due to various causes, such as mycotoxins (Aflatoxins), chemical carcinogens and viral infections (duck hepatitis B virus and leucosis virus) [3, 4, 15]. In the present case, we could not identify a possible cause. It is important to accumulate unique cases of such as CHCC to investigate the histogenesis and etiology. The present CHCC is the first reported case in the Yellow-headed Amazon species.

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