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Alpha-fetoprotein in serum and tumor tissues in dogs with hepatocellular carcinoma

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Abstract. Serum alpha-fetoprotein (AFP) concentrations were measured before and after surgical removal of tumor masses in four dogs with hepatocellular carcinoma (HCC). Localization of AFP was also examined immunohistochemically in tumor tissues. In three cases, the serum AFP concentration was 10 to 20 times higher than that of normal dogs. One to two months after surgery, the serum AFP concentration had decreased to normal range. AFP was localized in the tumor tissues in these three cases. One case, which had a low serum AFP, did not show AFP localization in tumor tissue.

Key words: Alpha-fetoprotein; canine hepatocellular carcinoma.

In humans, the serum level of alpha-fetoprotein (AFP), is used for the early diagnosis of hepatocellular carcinoma,^{1–4,7} in addition to blood chemistry data, radiography, ultrasonography, and computed tomography (CT). Lowseth et al. have assayed serum AFP concentrations associated with various canine tumors using enzyme-linked immunosorbent assay (ELISA) for human AFP.⁸ The results were variable and thus inadequate for diagnostic

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use. Yamada et al. previously developed an ELISA for canine AFP to investigate changes in serum AFP in newborn puppies.¹³ Subsequently, they found that, as in humans, AFP concentrations are elevated in canine tumors, especially in hepatocellular carcinoma (HCC).¹² In those reports, serum AFP was mildly elevated in some dogs with hepatic and nonhepatic diseases, even in those without tumors. A correlation between histopathology, immunohistochemistry, and serum AFP concentration in canine HCC has not been reported. This article reports relationship between pre- and postoperative serum AFP and the histopathologic and immunohistochemical features of the resected tumor in 4 dogs with HCC. This is the first report to demonstrate AFP distribution in tissues of canine HCC patients by using an anticanine AFP antibody.

All four cases studied in this report were seen at the Dobutu Medical Center. For clinical evaluation, complete blood cell counts were performed using an automatic blood

Table 1. Physical examination findings of 4 dogs with hepatocellular carcinoma at Dobutsu Medical Center.

	Case 1	Case 2	Case 3	Case 4
Breed	Shiba Inu	Mongrel	Shepherd Dog	Mongrel
Sex	male	spayed female	female	female
Age (year)	11	14	12	11
Body weight (kg)	7.8	9.0	26.7	19.8
Mass position	caudal lobe	right lobe	right lobe	left lobe
Mass size (cm)	7 × 8	10 × 15	30 × 40	15 × 15
Metastasis by the gross examination	no	no	no	other lobe
Histopathologic diagnosis*	well-differentiated HCC	well-differentiated HCC	poorly-differentiated HCC	well-differentiated HCC
AFP (ng/ml)	932	1,488	174	1,312
Survival time†	18 mo	29 mo	12 mo	7 mo

* HCC = hepatocellular carcinoma. † After extirpation of mass (mo = month).

cell counter.^a Blood chemistry was examined by an auto dry-chemical analyzer.^b Serum AFP concentrations were determined using a previously described sandwich ELISA for canine AFP.¹² Briefly, 96-well microplates (flat bottom) were coated with rabbit anticanine AFP IgG to capture AFP. AFP purified from canine amniotic fluid was used as a positive control.¹³ For detection of antibody-bound AFP, rabbit anticanine AFP IgG labeled with N-hydroxysuccinimidobiotin^c was used. Peroxidase-conjugated streptavidin^d and chromogen 2',2' azino-bis (3-ethylebenzthiazolin-6-sulfonic acid)^e were used for detection of biotin. Absorbance values of the samples and control were measured at 405 nm using a microplate reader^e. Resected tumor tissues were fixed in 10% phosphate-buffered formalin and sent to a commercial company for tissue sectioning. Classification of canine HCC followed World Health Organization (WHO) standards.⁶

For detection of AFP in tissue sections, a small piece (1 × 1 × 0.5 cm) of the resected tumor mass was frozen in liquid nitrogen. The frozen tissue was sectioned at 6 μm thicknesses with a Coldtome^f, and the section was placed on a glass slide. Endogenous peroxidase was inactivated by immersing in methanol containing 0.3% hydrogen peroxide for 10 minutes. Endogenous biotin was blocked using a biotin blocking kit.^g The tissue section was stained using an avidin-biotin complex method with biotinylated rabbit antidog AFP IgG,¹³ peroxidase-conjugated streptavidin^g and diaminobenzidine.^h Sections were counterstained with Lilly-Mayer hematoxylin.

Radiographic or ultrasonographic examinations showed masses in the liver, which were surgically removed. HCC was diagnosed by histopathologic examination. The histories and physical examination findings from those cases are shown in Table 1. Clinicopathologic findings are shown in Table 2. Case 1, which had been treated at another clinic for chronic hepatitis, was referred to the Medical Center with diarrhea of 2 months' duration, weight loss from 21 to 17.8 kg, and occasional shivering. Our physical examination revealed slightly pale mucous membranes, mild dehydration, and emaciation. Lateral and ventrodorsal radiographic images revealed a mass in the caudal liver. An ultrasonographic examination also de-

tected a mass in the hepatic parenchyma with a distinct echo pattern but without evidence of enlargement of the gall bladder or the bile duct. Cysts with unevenly echo-free cavities were also observed. The baseball-sized mass in the caudate lobe was surgically removed; there were no apparent metastases in the other hepatic lobes or in the greater omentum. The course of recovery was favorable; however, 12 months after surgery, the AFP concentrations and liver enzymes became elevated. The owner refused further diagnostic testing. Case 2 was presented to the Medical Center with symptoms of loss of vigor, occasional staggering, and uneven appetite of 2 weeks' duration. Slightly pale mucous membranes, mild dehydration, and mild emaciation were noted on physical examination, along with a cardiac murmur (grade III to IV) of the mitral valve. A simple radiograph showed a 15-cm mass at the center of the upper abdominal cavity. On ultrasonography, there was no enlargement of the gall bladder or the bile duct. A cystic mass was found with a pedunculated hyperechoic tissue mass extending into the cyst. The mass was surgically determined to originate in the right liver lobe with no obvious metastasis. A portion of the right lobe was resected, including a round tumor mass of 10 × 15 cm that was partially adhered to the greater gastric curvature, the small intestine, and the pancreas. The course of recovery was favorable, and the dog was released from the hospital after 6 days. Twenty-nine months after surgery, the dog died of renal failure. Permission for necropsy was denied. Case 3 was presented to the hospital with acute abdominal bloating of 4 days duration. The dog had a good appetite and was alert but slightly emaciated and exhibited a cardiac murmur (grade III to IV). A simple radiographic examination revealed a mass in the caudal liver. An ultrasonographic examination revealed a large echo-free cystic lesion of 30 × 40 cm in the liver parenchyma, as well as diffuse, cystic, and low-echo lesions. There were no apparent metastases in the other lobes, and the right lobe was entirely resected together with these masses. The mass contained multilocular cysts filled with approximately 1.2 ml of bloody serous fluid. The dog was released from the hospital after 7 days. The dog was seen again 2 weeks postsurgery. The dog died in a traffic

Table 2. Clinicopathologic findings immediately before and after the surgical removal of hepatocellular carcinoma in 4 dogs at Dobutsu Medical Center.

Parameter	Reference ranges	Case 1			Case 2			Case 3		Case 4	
		Before*	After†	One year‡	Before*	After†	One year‡	Before*	After†	Before*	After†
PCV (%)§	37–55	34	30	N/A	33	22	N/A	34	37	50	47
WBC (10 ³ /μl)§	60–170	133	N/A	N/A	121	317	N/A	493	259	139	106
T-Cholesterol (mg/dl)**	84–287	>400¶	188	N/A	235	172	N/A	164	193	151	253
T-Bilirubin (mg/dl)**	0–0.3	0.3	0.6	N/A	0.2	0.2	N/A	0.2	0.2	0.2	0.2
AST (IU/L)###	0–41	96	18	396	54	N/A	16	169	10	42	56
ALT (IU/L)###	0–123	592	146	1,000	107	N/A	30	482	151	734	677
ALP (IU/L)###	0–132	>500	>500	>500	>500	N/A	74	263	213	>500	N/A
T-Protein (g/dl)**	5.3–7.9	7.7	7.5	N/A	5.1	N/A	N/A	7.5	7.2	8	N/A
Albumin (g/dl)**	2.3–3.6	2.2	N/A	N/A	N/A	N/A	N/A	1.6	N/A	N/A	N/A
AFP (ng/ml)##††	<70¶	932	208	1,036	1,488	351	138	174	156	1,312	333

* Before extirpation of mass.

† Five to seven days after extirpation of mass.

‡ One year after extirpation of mass.

§ Measured by Celltac α (MEK-6258, NIHON KOHDEN), reference ranges were determined with 30 clinically healthy dogs.

|| N/A = not measured.

¶ > = over; < = under.

AST = aspartate aminotransferase; ALT = alanin aminotransferase; ALP = alkaline phosphatase; AFP = alpha-fetoprotein.

** Measured by SPOTCHEM sp-4410 (ARKRAY, Inc.), reference ranges were determined with 30 clinically healthy dogs.

†† AFP was measured by ELISA for canine AFP, reference range was determined with 20 clinically healthy dogs.

accident 1 year after surgery with no necropsy. Case 4 was presented to the hospital with sustained abdominal bloating, loss of appetite and vigor, with gradual weight loss. A simple radiograph showed a mass in the caudal liver, and an ultrasonographic examination revealed a tumor mass with a distinct echo pattern in the hepatic parenchyma. The mass was a cyst with an echo-free cavity. In addition, a solid mass of 15 cm diameter was found at the caudal rim of the left lobe. Gross examination through an abdominal median incision revealed a large mass on the caudal rim of the left lobe and disseminated metastases in the parenchyma of the other lobes. Only the large mass in the left lobe was resected. The dog was discharged from the hospital after 7 days and remained in good condition for the subsequent 4 months. The dog was reported dead 7 months after surgery, but was not presented for necropsy.

Repeated measurements of serum AFP concentrations were taken preoperation, postoperation and 1 year later, if the dog was available, using an ELISA¹² developed for canine AFP. Serum AFP concentrations were elevated before surgery in 3 out of 4 cases (Cases 1, 2, and 4), which were all diagnosed as well-differentiated HCC (Table 1). The AFP concentration dropped rapidly in these three cases after surgical removal of the tumor masses, returned to a normal range within a few months postoperation and remained low subsequently (Fig. 1). The AFP levels were increased in cases 1 and 2 at 12 months and 13 months postoperation respectively. Case 3 diagnosed as a poorly differentiated hepatocellular carcinoma had a presurgical serum AFP level within the normal range (Table 2). Case 1 was well-differentiated HCC. The tumor mass was composed of mildly pleomorphic hepatocytes that were arranged in irregularly thickened trabeculae. In areas, neo-

plastic hepatocytes also formed crude acini. The remaining hepatic parenchyma contained multiple dilated, blood-filled cavernous spaces (data not shown). Case 2 was well-differentiated trabecular HCC. The neoplastic cells were relatively small with clear cytoplasm, but they strongly resembled normal hepatocytes. They were arranged in cords (Fig. 2a). Case 3 was poorly differentiated HCC. The highly pleomorphic pale-staining tumor cells formed cords or solid nests lined with endothelial cells. Bile canaliculi were present in the tumor mass (Fig. 2b). Case 4 was well-differentiated HCC. The tumor cells retained a close resemblance to normal hepatocytes, but they showed evidence of invasive growth into the surrounding connec-

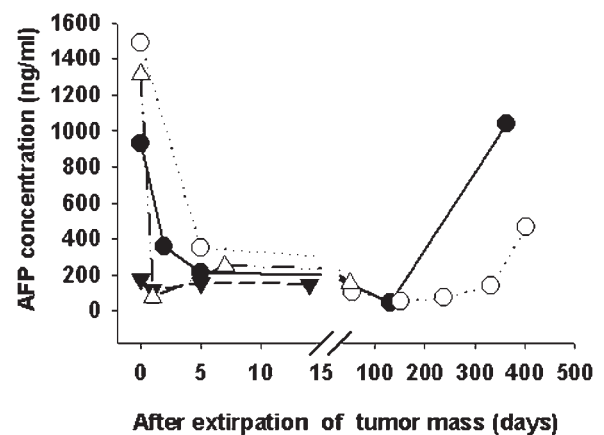


Figure 1. Changes in serum AFP concentration after surgical extirpation of tumor masses in cases 1 (closed circles), 2 (open circles), 3 (closed triangles), and 4 (open triangles) of hepatocellular carcinoma in dogs.

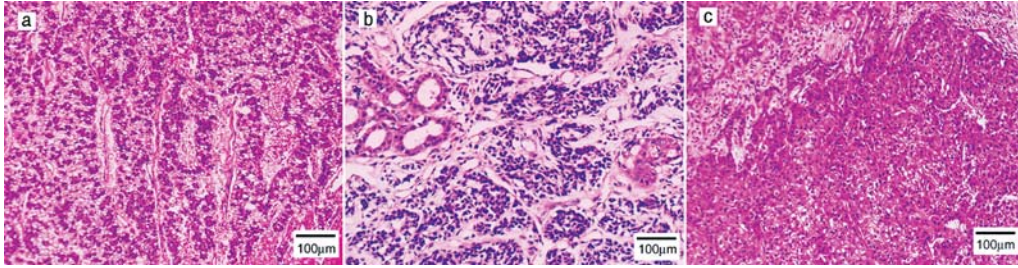


Figure 2. Histopathologic examinations showing **a** and **c** well-differentiated HCC from cases 2 and 4, **b** poorly differentiated hepatocellular carcinoma (HCC) from case 3, described in more detail in the text. HE stain.

tive tissue stroma (Fig. 2c). Significantly, intense AFP staining was detected in cases 1, 2, and 4 (Fig. 3a, 3b, 3c; data of case 1 not shown). However, AFP was not observed in Case 3 (Fig. 3d). The distribution of AFP was not homogenous in the tissues. AFP was observed in the cytoplasm of hepatic tumor cells (Fig. 3c).

HCC in humans often develops unnoticed until detected at a regular check-up or by close examination.⁷ The same applies to dogs, in which the tumor is usually well developed by the time the owner notices any abnormal signs.⁸ No characteristic symptom(s) are associated with HCC other than loss of appetite, sluggishness, and abdominal bloating. Serum chemistry results are usually within normal limits except for elevated liver enzymes. In addition to radiographic and ultrasonographic examinations and CT scanning, detection of tumor markers, including AFP, is important for

the diagnosis of human HCC. In animals, diagnosis is generally made by blood chemistry profiling and radiographic and ultrasonographic examinations. However, by the time tumor masses become detectable by these examinations, the tumor is likely to be well developed. The present 4 cases were all presented to the hospital when the owners noticed abnormalities. Serum alkaline phosphatase concentrations were higher than the normal limit in 3 out of the 4 cases, and alanin aminotransferase was also very elevated. Radiographic and ultrasonographic examinations revealed masses of 6 to 30 cm diameter, suggesting the presence of a hepatic tumor, possibly HCC. Because liver enzymes can also be elevated in other hepatic diseases, these parameters cannot give a definitive diagnosis.⁸ In addition, a tumor mass has to reach a certain size to be reliably detected by radiography or sonography. In humans, high proficiency in

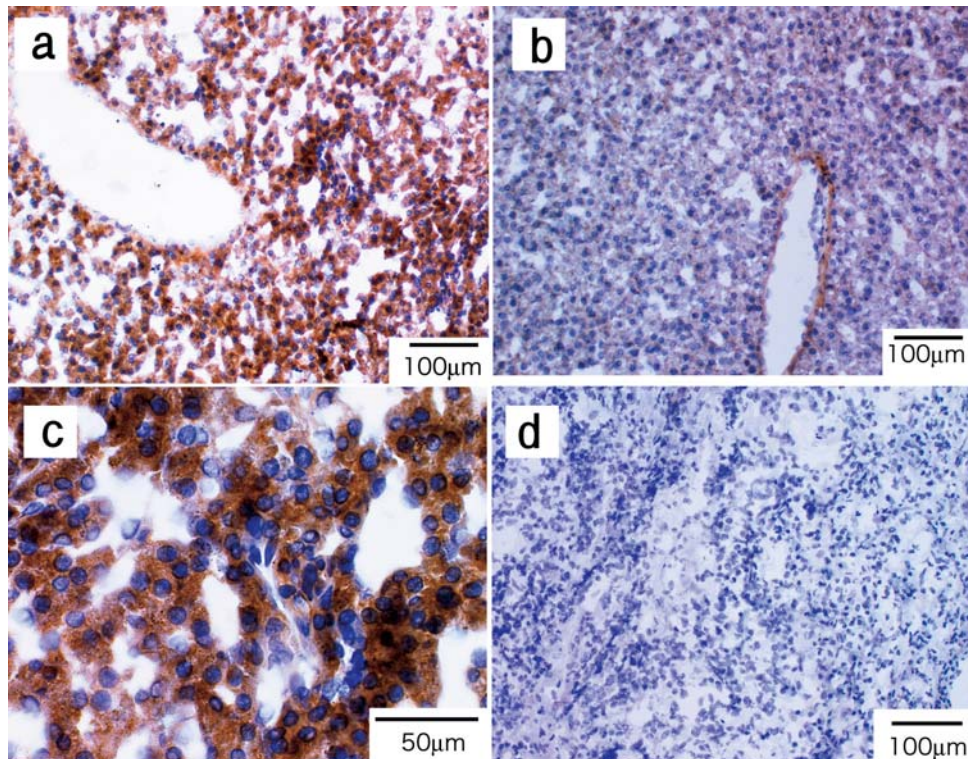


Figure 3. Results of the avidin-biotin complex method using a biotinylated anticanine AFP antibody. **a** and **b**, Positive peroxidase signals observed in the well-differentiated HCC from cases 2 and 4, **c** high magnification of **a**, **d** negative peroxidase signals in the undifferentiated HCC from case 3.

ultrasonography is required for detection of a 2-cm-diameter mass, at which time the serum AFP concentration can already be elevated to nearly 1,000 ng/ml.⁷ In this report, the change in serum AFP concentration before and after surgical resection of the tumor mass was followed in an attempt to establish the use of serum AFP concentration as an early marker of HCC. Serum AFP concentrations before surgery were significantly higher in all cases except the one with undifferentiated HCC. In humans, it is known that the serum AFP concentration increases with the size of tumor masses, although no such correlation was found in this study. This may have resulted from the increased rate of necrosis at the center of the tumor masses in the present cases. Guillouzo et al.⁵ have shown that AFP is synthesized during the G₀ or G₁ phases in hepatic cells of neonatal rats. Although differences between normal and cancer cells have to be considered, actively dividing tumor cells may cause an elevated AFP concentration in serum even when the tumor mass is still small. These 4 cases were in the later stages of HCC, but serum AFP concentrations might have been elevated in the earlier stages of carcinogenesis if they had been examined. Serum AFP concentrations of three cases were decreased after removal of tumor masses. However, in cases 1 and 2, serum AFP concentrations were increased again 1 year after extirpation of mass. It was suspected that they had metastasis or relapse of the tumor, although they were not examined. Additionally, this indicates that reactivation of a tumor may be monitored by changes in the serum AFP concentration. Immunohistochemical examinations indicated increased AFP synthesis in the tumor tissues from the cases with elevated concentrations of serum AFP. AFP was not detected at the center of the tumor masses occupied with necrotizing tissues but was highly increased in the margin of tumor tissue (data not shown). It has also been shown that the serum AFP concentration increases when normal hepatocytes are actively dividing,⁹⁻¹¹ and a moderate increase in serum AFP concentration is also reported in hepatic dysfunction or tumors other than HCC.¹² Therefore, quantification of serum AFP may not be used per se for a definitive diagnosis of HCC. In a previous study with a small population, 90% of dogs (19/21) with hepatic disease but without tumors were shown to have AFP concentrations of under 500 ng/ml, whereas 92% of tumor-bearing dogs (50/54) without hepatic tumors had concentrations of under 1,000 ng/ml.¹² When combined with the results of other detailed examinations, the measurement of the serum AFP concentration may be a useful biochemical marker for the diagnosis of HCC. On the other hand, a lower AFP concentration was seen in 1 case with a poorly differentiated HCC that was thought to have a highly mosaic ability to produce AFP.² Further studies are required to determine the relationship between the types of HCC and AFP synthesis and HCC that is unaccompanied by increased synthesis of AFP.

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Sources and manufacturers

- a. Celltac α , MEK-6258, NIHON KOHDEN, Tokyo, Japan.
- b. SPOTCHEM sp-4410, Arkray, Kyoto, Japan.
- c. Sigma-Aldrich Biotechnology, Tokyo, Japan.
- d. Dako Japan Ltd, Kyoto, Japan.
- e. Model 550 BIORAD, CA, USA.
- f. CM-501, Sakura Finetek Ltd, Tokyo, Japan.
- g. Nichirei HISTOFINE, Nichirei, Tokyo, Japan.
- h. DAB KIT, Nichirei HISTOFINE, Nichirei, Tokyo, Japan.

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