Canine Serum Thermostable Alkaline Phosphatase Isoenzyme From a Dog With Hepatocellular Carcinoma

Yu-ichi FUKUI¹⁾, Jun SATO^{1,2)}, Reeko SATO¹⁾, Jun YASUDA¹⁾ and Yoshihisa NAITO¹⁾

¹⁾Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Morioka 020-8550 and ²⁾Tomizawa Animal Hospital, Sendai 982-0032, Japan

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ABSTRACT. A dog histopathologically diagnosed with hepatocellular carcinoma (HCC) showed very high serum alkaline phosphatase (ALP) activity. A supernatant of ascitic fluid and tumor tissue extracted from the dog also showed much higher ALP activity than normal. ALP isoenzyme analysis of samples was performed using polyacrylamide gel disk electrophoresis, and a wide, broad abnormal band was observed. By various treatments, the abnormal band showed thermostability, which is a characteristic of tumor-associated ALP that has only been reported in humans. The thermostable ALP isoenzyme was not found in sera from 39 dogs with several types of tumor that originated from the liver, except for HCC, nor was it found in 10 dogs with hepatic diseases that did not include hepatic tumors. The thermostable ALP isoenzyme seemed to be associated with canine HCC.

KEY WORDS: canine hepatocellular carcinoma, polyacrylamide gel disk electrophoresis, thermostable alkaline phosphatase isoenzymes.

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Alkaline phosphatase (ALP) is a membrane-bound glycoprotein that is present in many tissues [11]. Four isoenzymes of ALP have been identified in humans, tissue-nonspecific ALP (TNALP), intestine ALP (IALP), placenta ALP (PALP), and germ cell ALP (GCALP) [2]. Tissue-nonspecific ALP has been further classified into bone ALP (BALP) and liver ALP (LALP) based on the differences in their sugar chain structures [8]. In sera from humans, BALP, LALP, and IALP are constantly present. On the other hand, PALP is observed only during pregnancy [8, 11]. However, PALP and GCALP are sometimes present in sera from patients with malignant neoplasia, such as a lung tumor, ovary tumor, seminoma, or hepatic tumor [2, 8].

Three ALP isoenzymes, which are recognized as BALP, LALP, and corticosteroid-induced ALP (CALP), have been identified in canine serum [15, 17]. Corticosteroid-induced ALP is a canine-specific ALP isoenzyme that is induced by excess endogenous and exogenous glucocorticoid [16]. Its amino acid sequence is the same as IALP, but there is a difference in their sugar chain structure [13]. Placenta ALP and GCALP have not been found in canine tissues while TNALP is expressed in the canine placenta instead of PALP, which is expressed in humans [9]. The absence of other types of ALP isoenzyme in canine serum might be attributed to their short half-lives [17].

On the other hand, several tumor-associated abnormal ALP isoenzymes have been reported in sera from human cases. These were not only variant types of PALP and GCALP, called Regan and Nagao isoenzyme, respectively [17], but also IALP with a variant sugar chain structure, called Kasahara isoenzyme [8]. Furthermore, abnormal ALP with a GPI-anchor that contributes to binding with the cell membrane [2], called high-molecular ALP [8], has been detected in patients with hepatic tumors. However, there are currently no reports about tumor-associated abnormal ALP isoenzymes in dogs [17].

In the present study, we observed a tumor-associated abnormal ALP isoenzyme in a tumor-bearing dog with high serum ALP activity using a commercially available polyacrylamide gel disk electrophoresis kit that has been proven useful in canine serum ALP isozyme analysis [6].

A thirteen-year-old female Shiba dog with abdominal enlargement was brought to the Veterinary Teaching Hospital of Iwate University (VTHIU) for a thorough examination. The dog showed anorexia, diarrhea, and vomiting. A palpable intraabdominal mass was detected, and was observed radiographically and echographically caudal to the liver. In addition, a large amount of ascitic fluid had manifested. Lung metastasis was not confirmed by thoracic radiography. Moderate anemia and neutrophilia were revealed by complete blood count. Serum ALP activity was 9820 IU/l, and serum alanine transaminase and gamma-glutamyl-transferase activities were 285 IU/l and 55.3 IU/l, respectively.

On exploratory laparotomy, a neoplasm was detected originating from the quadrate lobe of the liver with diffuse metastasis in the abdominal cavity. The ascitic fluid contained a large amount of blood exuded from diffused metastatic lesions. The attending doctor concluded that the neoplasm was very malignant and the prognosis of the dog was poor. The patient dog was euthanized with the owner's agreement. As a result of necropsy, a metastatic lesion was also observed in the lung. Histopathologically, the neoplasm was diagnosed as hepatocellular carcinoma (HCC) with diffuse necrosis.

The dog's serum, supernatant of ascitic fluid and tumor tissues from primary liver carcinoma and metastatic lesions of other organ were examined by ALP isoenzyme analysis. In addition, sera were collected from 20 healthy dogs belonging to a dog-training school, and parenchymal liver and cancellous bone tissues were taken from three adult healthy beagles after euthanasia in order to compare the val-

ues of ALP activity and isoenzyme with those of the patient dog. Sera was also collected from 39 dogs diagnosed malignant tumors that did not originate from the liver and 10 dogs diagnosed with hepatic diseases, except for hepatic tumor, showing high serum ALP activity (>1000 IU/l). These dogs were presented at VTHIU from January 2004 to March 2005. The 39 tumor cases included lymphoma ($n=11$), mast cell tumor ($n=7$), primary lung tumor ($n=3$), malignant histiocytoma ($n=3$), transitional cell carcinoma ($n=2$), hemangiosarcoma ($n=2$), perianal adenocarcinoma ($n=2$), mammary gland adenocarcinoma with lung metastasis ($n=2$), osteosarcoma, prostate gland adenocarcinoma, squamous cell carcinoma, fibrosarcoma, multiple myeloma, seminoma, and sarcoma NOS. The 10 cases of hepatic diseases included chronic congestion from congestive heart failure ($n=2$), steroid hepatopathy from excess glucocorticoid ($n=2$), biliary tract disorder ($n=2$), cirrhosis, and miscellaneous disorders with high alanine transaminase activities ($n=3$).

Each tissue sample was suspended in physiological saline (2 ml/g), homogenized by ultrasound homogenizer, and centrifuged at 3,000 rpm for 10 min to separate the supernatant. Each supernatant was treated with Triton X-100 (final concentration, 0.2%) (Polysciences, Inc., U.S.A.) and phosphatidylinositol-phospholipase C (PI-PLC, final concentration 0.02 U/ml) (Funakoshi Co., Ltd., Japan). All samples were stored at -20°C until analysis.

ALP activity was assayed using a commercial ALP kit (Liquitech ALP, Roche Diagnostics Co., Ltd., Japan) with an automatic analyzer (Automatic Analyzer 7060, Hitachi Ltd., Tokyo, Japan). Quantitative measurement of ALP isoenzyme activity (BALP, LALP, and CALP) was performed by the method previously reported by Syakalima *et al.* [15].

Polyacrylamide gel disk electrophoresis was performed using a commercially available kit (AlkPhor System, Jokoh Co., Ltd., Japan) according to the manufacturer's instructions. The collected sera and supernatant samples were used for electrophoresis. The extracts from the livers and bones of the healthy dogs were used as LALP and BALP controls, respectively. In the present study, the migration patterns of ALP isoenzyme in the sera from the healthy dogs were regarded as the normal control.

Wheat germ agglutinin (WGA) has been reported to bind sialic acid and N-acetylglucosamine in the sugar chains of

BALP and CALP and result in precipitation of these isoenzymes [13]. The samples were mixed with the same volume of WGA (5 mg/ml in distilled water) (Wako Pure Chemical Industries, Ltd., Japan). The mixture was incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 10 min. Its supernatant was used in ALP quantitative analysis and electrophoresis to identify ALP isoenzymes with a specific sugar chain sensitive to WGA; these were normally BALP and CALP [14].

Levamisole hydrochloride (Wako Pure Chemical Industries, Ltd., Japan) was added to samples or substrate buffer at final concentrations of 4.2 mM to inhibit TNALP activity [5].

PI-PLC was added to samples at a final concentration of 0.2 U/ml, and the samples were then incubated at room temperature overnight. This mixture was used for electrophoresis to detect the presence of high molecular ALP because PI-PLC cuts off GPI-anchor and makes its migration distance increase [12].

Samples were mixed with the same volume of 10 mM MgCl_2 solution, heated at 65°C for 30 min in a water bath, and then cooled on ice. The heated samples were used for electrophoresis to detect the presence of thermostable ALP isoenzymes including PALP, GCALP, and their variants [2, 11].

The serum activities of LALP and CALP from the dog with HCC were 7527 IU/l, 2293 IU/l, respectively. The ALP isoenzyme activities from the supernatant of ascitic fluid were almost identical. The ALP isoenzyme activities from the tumor tissue extracts were much higher than those from normal liver extracts (Table 1).

In the electrophoretic migration patterns from the healthy dogs, an LALP band was sharply present on the anode side as a single line and the BALP and CALP bands were obscurely present on the cathode side (Fig. 1 Cont.). However, in the serum of the dog with HCC, a normal sharp LALP band and very wide, broad abnormal band were found (Fig. 1S). The supernatant of ascitic fluid showed the same migration pattern as the serum (Fig. 1A). The migration pattern of the tumor tissue extracts from both of primary and metastatic lesions also had the same abnormal broad band without an LALP band (Fig. 1 Tp and Tm). The abnormal band was resistant to all treatments, including WGA treatment, levamisole inhibition, PI-PLC treatment, and heat inactivation at 65°C for 30 min (Fig. 2).

Table 1. ALP isoenzyme activities of serum, ascitic fluid, and tumor tissue extracts from the dog with HCC

	The dog with HCC				Healthy dogs ^{a)}	
	Serum	Ascitic fluid	Primary tumor	Metastatic tumor	Serum ($n=20$)	Liver extract ($n=3$)
ALP (IU/l)	9820	8540	11830	5400	71.2 ± 4.5	855 ± 131
BALP (IU/l)	Not detected	Not detected	6490	1047	44.1 ± 4.5	$850 \pm 128^b)$
LALP (IU/l)	7527	6256	306	Not detected	23.0 ± 9.7	
CALP (IU/l)	2293	2284	5034	4353	4.1 ± 2.4	4.74 ± 2.93

a) ALP isoenzyme activities of sera and liver tissue extracts from healthy dogs were provided as a normal control. Data presented as means $\pm$ standard error of the mean (SEM).

b) Calculated as the total activity of BALP and LALP.

Fig. 1. Polyacrylamide gel disk electrophoresis of ALP isoenzymes from the dog with HCC. Extracts of normal liver (Liver) and bone (Bone) were presented as markers of LALP and BALP, respectively. Cont. shows the normal serum ALP isoenzyme pattern from a healthy dog. A broad abnormal band was observed in the serum (S), supernatant of ascitic fluid (A), and primary (Tp) and metastatic tumor tissue extracts (Tm) of the dog with HCC.

Fig. 2. Polyacrylamide gel disk electrophoresis of ALP isoenzymes from the serum of the dog with HCC (N. T.). The abnormal broad band was resistant to WGA treatment (+W), levamisole inhibition (+L), PI-PLC treatment (+P), and heat inactivation (+H). The normal serum ALP isoenzyme pattern from a healthy dog (Cont.) illustrates the normal migration distance of LALP.

Abnormalities, such as a decreased migration distance of the LALP band and abnormal bands, were also found in several sera from the other tumor cases and hepatic-diseased cases (Figs. 3 and 4). However, the migration distance of LALP was normalized by WGA and PI-PLC treatment, and the abnormal bands disappeared after heat inactivation. Electrophoresis showed no apparent relationship for these abnormalities among the types of diseases, severity and prognosis of the diseases, and ALP isoenzymes and other liver enzyme activities (data not shown).

Several reports have referred to the efficacy of serum ALP isoenzyme analysis on specific canine neoplasia, including osteosarcoma [1], mammary gland tumor [7], and lymphoma [18]. However, there was only one report about ALP isoenzyme patterns of serum and tumor tissue extracts from a tumor-bearing dog diagnosed with lymphoma with neoplastic infiltration of the liver, and this study used cellulose-acetate electrophoresis [4]. The report concluded that the abnormal ALP isoenzyme was identical to CALP. Therefore, the increase of ALP activity in the sera from tumor-bearing dogs was attributed to CALP activity induced by excess endogenous glucocorticoid resulting from stress reaction [17].

However, interestingly, we found an abnormal thermostable ALP isoenzyme in the dog with HCC in this study. The LALP activities in the serum and ascitic fluid of the dog

Fig. 3. Examples of decreased migration distance of the LALP band from a dog with a mast cell tumor (N. T.). The migration distance of LALP was normalized by WGA (+W) and PI-PLC treatment (+P), but not by levamisole inhibition (+L). The normal serum ALP isoenzyme pattern from a healthy dog (Cont.) illustrates the normal migration distance of LALP.

Fig. 4. Examples of thermolabile abnormal bands from a dog with hemangiosarcoma (N. T.). These abnormal bands were resistant to WGA treatment (+W), levamisole inhibition (+L), and PI-PLT treatment (+P); however, they disappeared after heat inactivation (+H). The normal serum ALP isoenzyme pattern from a healthy dog (Cont.) is also shown.

were high, but were low in the tumor tissue extracts. An LALP band was detected by electrophoresis in the serum and ascitic fluid, but not in the tumor tissue extracts. This means that the LALP did not originate in the tumor tissue.

In addition, the proportion of BALP and CALP activities in the tumor tissue extracts was higher than those in the serum and ascitic fluid. ALP isoenzymes precipitated by WGA are calculated as BALP [13], and we found these in extracts from not only bone but also many organs including the liver (data not shown). On the other hand, CALP measurement by levamisole inhibition is based on CALP's resistance to levamisole [5]. The abnormal isoenzyme was thought to be calculated as CALP activity because the abnormal band had the characteristics of resistance to levamisole inhibition based on the results of electrophoresis. Therefore, this isoenzyme was thought to originate from the tumor tissues.

Moreover, this isoenzyme was similar to PALP and GCALP with regard to its resistance to all treatments, especially heat inactivation at 65°C for 30 min. PALP and GCALP have only been reported in humans, and have unique properties including resistance to heating at 65°C for 30 min [2, 11]. This isoenzyme detected in this study might be identical to PALP or GCALP.

In this study, we could not detect the thermostable isoenzyme in the other tumor cases or hepatic-diseased cases although a decreased migration distance of the LALP band and abnormal bands were found in some of them. These abnormalities were not thought to be specific to malignant tumors because they appeared in non-tumor diseases and showed no relationship with the type of disease, severity, and prognosis. However, the decreased migration distance of the LALP band seemed to be associated with a GPI-anchor because the abnormality was normalized by PI-PLC treatment. The shortened LALP migration distance might be identical to that of high-molecular ALP reported in sera from patients of hepatic tumors or disease [12]. The thermostable abnormal bands were thought to be CALP with variant sugar chain structures because their characteristics were similar to those of CALP apart from migration distance. Changes in sugar chain structures have been reported in serum ALP from human patients with tumors and cirrhosis [3, 10]. In this study, a thermostable ALP isoenzyme was found in only one dog with HCC, and we could not detect this isoenzyme in either hepatic-diseased or other tumor-bearing dogs. Accordingly, this isoenzyme might be associated with highly malignant HCC. However, further studies are required to focus on the clinical properties of this isoenzyme in detail.

REFERENCES

1. Ehrhart, N., Dernell, W. S., Hoffmann, W. E., Weigel, R. M., Powers, B. E. and Withrow, S. J. 1998. *J. Am. Vet. Med. Assoc.* **213**: 1002–1006.
2. Fishman, W. H. 1990. *Clin. Biochem.* **23**: 99–104.
3. Fukushima, K., Hada, T., Higashino, K. and Yamashita, K. 1998. *Clin. Cancer Res.* **4**: 2771–2777.
4. Hoffmann, W. E. and Dorner, J. L. 1975. *Clin. Chim. Acta.* **62**: 137–142.
5. Hoffmann, W. E., Sanecki, R. K. and Dorner, J. L. 1988. *Vet. Clin. Pathol.* **17**: 66–70.
6. Itoh, H., Kakuta, T., Genda, G., Sakonju, I. and Takase, K. 2002. *J. Vet. Med. Sci.* **64**: 35–39.
7. Karayannopoulou, M., Koutinas, A. F., Polizopoulou, Z. S., Roubies, N., Fytianou, A., Saridomichelakis, M. N. and Kaldrymidou, E. 2003. *J. Vet. Med. A* **50**: 501–505.
8. Komoda, T. 1997. *Nihon-Iji-Shinpo* **3797**: 106–107.
9. Kramer, J. W. 1989. pp. 338–363. In: *Clinical Biochemistry of Domestic Animals*, 4th ed. (Kaneko, J. J. ed.), Academic Press, Inc., San Diego.
10. Lu, Y., Lu, Q. and Chen, H. L. 1997. *J. Exp. Clin. Cancer. Res.* **16**: 75–80.
11. Marshall, M. and Kaplan, M. D. 1972. *Gastroenterology* **62**: 452–468.
12. Sakai, T., Makita, A., Kondo, T., Miyake, K., Yamanaka, M., Komoda, T. and Sakagishi, Y. 1990. *Igaku-no-Ayumi* **155**: 741–742.
13. Sanecki, R. K., Hoffmann, W. E., Dorner, J. L. and Kuhlen-schmidt, M. S. 1990. *Am. J. Vet. Res.* **51**: 1964–1968.
14. Sanecki, R. K., Hoffmann, W. E., Hansen, R. and Schaeffer, D. J. 1993. *Vet. Clin. Pathol.* **22**: 17–23.
15. Syakalima, M., Takiguchi, M., Yasuda, J. and Hashimoto, A. 1997. *J. Vet. Med. A* **44**: 603–610.
16. Syakalima, M., Takiguchi, M., Yasuda, J. and Hashimoto, A. 1997. *J. Vet. Med. Sci.* **59**: 905–909.
17. Syakalima, M., Takiguchi, M., Yasuda, J. and Hashimoto, A. 1998. *Jpn. J. Vet. Res.* **46**: 3–11.
18. Wiedemann, A. L., Charney, S. C., Barger, A. M., Schaeffer, D. J. and Kitchell, B. E. 2005. *J. Small. Anim. Pract.* **46**: 185–190.