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Plasma-free amino acid profiles in dogs with hepatocellular carcinoma

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Abstract

Background: Metabolomic analysis using blood samples has been suggested to be useful for the early detection of cancer. Among metabolites, plasma-free amino acid (PFAA) profiles are potential diagnostic biomarkers for several diseases including cancer. However, the relationship between PFAA concentrations and liver tumors in dogs remains unknown.

Objective: To determine the characteristics of PFAA profiles of dogs with hepatocellular carcinoma (HCC) and correlated clinical features.

Animals: Thirty-four client-owned dogs diagnosed with HCC (n = 26) and benign liver diseases (n = 8) and 11 age-matched healthy dogs.

Methods: Prospective study using heparinized blood samples from fasted dogs. Plasma was deproteinized, and the concentrations of 21 amino acids were measured using an automated high-performance liquid chromatography amino acid analyzer.

Results: Plasma glutamic acid concentrations were significantly different among groups ($P < .0024$ after Bonferroni correction). Compared to healthy dogs, dogs with HCC and benign liver diseases had significantly higher concentrations of glutamic acid by post hoc analysis. However, no significant difference in the PFAA profiles of HCC and benign liver diseases were detected. In addition, preoperative and postoperative PFAA profiles of dogs with HCC were not significantly different.

Conclusions and Clinical Importance: Increased glutamic acid concentrations might play a role in the development or be a consequence of liver tumor formation. However, PFAA profiles of HCC could not be differentiated from those of benign lesions. In addition, glutamic acid concentrations did not change after surgical resection. These results indicate that PFAA profiles may not be useful biomarkers for detecting HCC in dogs.

KEYWORDS

biomarker, dog, liver tumor, metabolomics

Abbreviations: AAA, aromatic amino acid; BCAA, branched-chain amino acid; BTR, branched-chain amino acids to tyrosine ratio; HCC, hepatocellular carcinoma; PFAA, plasma-free amino acid; TAA, total amino acid.

1 | INTRODUCTION

Primary liver tumors are relatively rare, accounting for 0.6% to 1.3%[1] of all tumors in dogs. The most common primary liver tumor in dogs is hepatocellular carcinoma (HCC),[1-3] which is frequently found in older dogs. Although liver tumors generally cannot be diagnosed by clinical signs, blood examination, or abdominal radiography, they are easily detected using abdominal ultrasonography and computed tomography, resulting in an increase in the number of animals in which liver tumors are incidentally discovered at an advanced stage.[4] Unfortunately, advanced diagnostic imaging, including computed tomography, may not be readily accessible for use in dogs because of high cost; need for sedation or anesthesia, which is not desirable for older dogs or those with advanced disease; and, modalities and limited availability of such equipment in veterinary facilities. In addition, current diagnostic imaging may not be able to distinguish malignant tumor from benign changes. Moreover, early detection of liver tumors in dogs using current diagnostic methods is still challenging. Therefore, development of a noninvasive diagnostic method with high sensitivity and specificity would be valuable for the early detection and classification of pathological conditions in the liver.

Metabolomics is a powerful method that allows for the analysis of all metabolites in a biological system. In this way, metabolic changes associated with homeostatic disturbances in individual pathological conditions can be identified.[5,6] Recently, metabolomic analysis using peripheral blood or urine samples has been developed as a promising approach for identifying several diseases, including cancer.[7] The hypothesis underlying the detection and profiling of changes in metabolites is based on the expectation that they will offer new perspectives not only for the processes of disease development but also for the identification of diagnostic biomarkers. Moreover, metabolic variations may be indicative of the biochemical status associated with individual diseases.[8]

Among the various metabolites, amino acids are the most suitable candidates for focused metabolomic analyses because they play crucial physiological roles as both substrates and regulators in many metabolic pathways.[8-10] Measurements of plasma concentrations of free amino acids, which can be obtained easily from patients, can provide useful information concerning disease states because they are linked to all organ systems. Alterations of plasma-free amino acid (PFAA) profiles are associated with metabolic changes induced by specific diseases[7] that affect protein metabolism.[11] Recent studies have reported that PFAA concentrations are altered in various diseases in humans, including cancer.[7,12-15] These amino acid imbalances are influenced by changes in protein metabolism, which are related to the development of disease.[11,13,14] Therefore, PFAA profiles may be useful biomarkers for disease diagnosis. In veterinary medicine, alterations of PFAA concentrations have been reported as potentially useful biomarkers for several tumors, including malignant mammary tumors, melanomas of the oral cavity, lymphomas, and brain tumors.[10,16-18] However, to our knowledge, no studies have investigated the association between PFAA concentrations and liver tumors in dogs. Therefore, an aim of our study was to investigate the changes in PFAA profiles in dogs with HCC in comparison to healthy control dogs and dogs with benign liver diseases. Furthermore, we sought to correlate any significant changes in PFAA profiles with other laboratory findings, HCC size, and completeness of excision after surgery.

2 | MATERIALS AND METHODS

2.1 | Study population

A total of 34 dogs with focal liver lesions examined at a referral veterinary teaching hospital from November 2016 to December 2018 were prospectively evaluated. The diagnosis of focal liver lesions was based on histopathological examination after surgery. All histopathological examinations were performed by an American College of Veterinary Pathology board-certified pathologist (Dr. Yumiko Kagawa). Eleven healthy age-matched control dogs were included in the study. The heath of control dogs was based on normal physical examination findings, normal CBC and serum biochemistry panel, and the absence of any known tumors. Medical records of all included dogs were reviewed for demographic information (age, body weight, breed, and sex) and laboratory findings on the date of blood collection, as well as tumor data, including computed tomography information on HCC size and completeness of excision after surgical resection. All of the animal procedures were approved by the Institutional Animal Care and Use Committee, and informed consent was obtained from all owners of the dogs involved in the study.

2.2 | Blood collection and processing

The dogs were fasted for 12 hours before blood collection. Venous blood was collected in heparin-containing tubes from control dogs and dogs diagnosed with HCC. Samples were collected preoperatively and 3 to 6 months postoperatively. After blood collection, the heparinized blood samples were centrifuged immediately at 1500*g* for 5 minutes at 4°C. After centrifugation, plasma was promptly removed and frozen at −80°C until PFAA measurement. On the day of PFAA measurement, frozen plasma was thawed, mixed with equal volumes of 5% (wt/vol) trichloroacetic acid, and centrifuged at 1000*g* for 15 minutes at 4°C to remove precipitated proteins and obtain the supernatant.

2.3 | PFAA measurement

Analysis of the PFAA concentrations from the dogs was performed using an automated high-performance liquid chromatography amino acid analyzer (L-8900; Hitachi, Tokyo, Japan). Amino acids were separated by ion exchange chromatography and detected spectrophotometrically after a post-column reaction with ninhydrin reagent. The concentrations of 21 naturally occurring amino acids (alanine, arginine, asparagine, citrulline, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, taurine, threonine, tryptophan, tyrosine, and valine) were measured in the analysis. The total amino acid (TAA) concentrations were

calculated as the sum of the concentrations of these 21 amino acids. All amino acid concentrations were expressed in nmol/mL.

Fischer's ratio is defined as the molar concentration ratio of total branched-chain amino acids (BCAAs), calculated as the sum of the leucine, valine, and isoleucine concentrations, to total aromatic amino acids (AAAs), calculated as the sum of the phenylalanine and tyrosine concentrations. This ratio was calculated from the PFAA concentrations, as was the branched-chain amino acids to tyrosine ratio (BTR), which is a simpler calculation and can be used instead of Fischer's ratio.[19,20] These ratios were used to evaluate liver function and severity of liver damage, and decreases in these 2 variables can reflect increasing severity of liver damage.[21,22]

2.4 | Statistical analysis

Continuous variables, including age, body weight, lesion size, and PFAA concentrations, were assessed for normality using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) and the Kruskal-Wallis test were used to compare normally and non-normally distributed data among groups of dogs with malignant and benign liver lesions and age-matched control dogs. These tests were followed by the Tukey honestly significant difference and Steel-Dwass test for post hoc analysis, respectively. Matched-pair analyses, including the paired *t* test and the Wilcoxon matched pairs signed rank test, were used to analyze normally distributed and non-normally distributed PFAA concentrations between the preoperative and postoperative groups, respectively. The data were expressed as medians and ranges for age, body weight, serum liver enzyme activities of dogs with HCC, HCC size, and non-normally distributed PFAA concentrations. The data were expressed as means and standard deviations for normally distributed PFAA concentrations. Categorical variables, including sex and serum liver enzyme activities, were analyzed using Fisher's exact test or the chi-square test and presented as numbers and percentages.

Pearson's and Spearman's correlation analyses were used to determine the relationship between significant PFAA concentrations of dogs with HCC and serum liver enzyme activities as well as lesion size. Bonferroni correction was applied to account for multiple comparisons of PFAAs by ANOVA and Kruskal-Wallis test. Statistical analyses were performed using commercial software (JMP Pro, version 14.0.0; SAS Institute Inc, Cary, North Carolina). A value of $P < .05$ was considered statistically significant ($P < .0024$ after Bonferroni correction).

3 | RESULTS

3.1 | Dogs

During the study period, 46 dogs were identified with liver masses and were surgically treated. Of these, 26 dogs (13 males and 9 females) were diagnosed with HCC, and 8 (3 males and 5 females) were diagnosed with benign liver diseases, including 3 with cholangiocellular adenoma, 2 with nodular hyperplasia, and 1 case each of portal vein hypoplasia, hepatic cyst, and hematoma. The other 12 dogs were excluded from the study because of histopathologic results indicating metastatic lesions, multifocal liver lesions with both benign and malignant histopathologic findings, and comorbidities.

The breed distribution of HCC dogs was as follows: 3 Chihuahuas, 3 Miniature Schnauzers, 2 Beagles, 2 Miniature Dachshunds, 2 Mongrels, 2 Shiba Inus, 2 Shih Tzus, 2 Toy Poodles, 2 Yorkshire Terriers, and 1 each of Border Collie, Boston Terrier, Golden Retriever, Pekingese, Shetland Sheepdog, and Welsh Corgi. Age at diagnosis of HCC ranged from 7 to 16 years, with a median of 12 years. Body weight ranged from 1.7 to 32 kg, with a median of 7.7 kg.

Breeds of dogs with benign liver diseases included 3 Yorkshire Terriers and 1 each of Maltese, Miniature Dachshund, Chihuahua, Toy Poodle, and Welsh Corgi. Age at diagnosis of benign liver diseases ranged from 6 to 16 years, with a median of 11.5 years. Body weight ranged from 2 to 7.6 kg, with a median of 3.1 kg.

Eleven healthy age-matched control dogs were examined, including 5 males and 6 females. Breeds included 6 Beagles, 3 Miniature Dachshunds, 1 Mongrel, and 1 Yorkshire Terrier. The age of the control dogs ranged from 7 to 15 years, with a median of 10 years. Body weight ranged from 2.4 to 14.4 kg, with a median of 12 kg.

Regarding the study population, no significant differences in age at diagnosis were identified among the groups with benign lesions, malignant liver lesions, and controls ($P = .6302$). In addition, no significant differences in sex were found among the groups ($P = .6967$). However, body weight was significantly different among the HCC, benign liver disease, and control groups ($P = .0125$).

3.2 | Serum liver enzyme activities of dogs with HCC

All dogs with HCC had increased alkaline phosphatase activity (median, 3310.5 IU/L; range, 358-3501 IU/L; reference range, 47-254 IU/L). Twenty-one of 22 dogs (95%) had increased alanine aminotransferase (ALT) activity, with the remaining dog (5%) having ALT activity within the reference range (median, 260 IU/L; range, 67-1001 IU/L; reference range, 17-78 IU/L). Five of 11 dogs (45%) had increased aspartate aminotransferase (AST) activity, whereas 6 of 11 dogs (55%) had AST activity within the reference range (median, 42 IU/L; range, 21-1001 IU/L; reference range, 17-44 IU/L). Four of 10 dogs (40%) had increased gamma-glutamyl transferase (GGT) activity, whereas 6 of 10 dogs (60%) had GGT activity within the reference range (median, 10 IU/L; range, 2-159 IU/L; reference range, 5-14 IU/L).

3.3 | Size of HCC and completeness of excision after surgery

All dogs with HCC underwent complete surgical resection. Based on computed tomography results, the transverse diameter of HCC size ranged from 2.5 to 12.9 cm, with a median of 6.2 cm.

3.4 | PFAA concentrations

A comparison of the PFAA concentrations among the dogs with HCC, benign liver disease, and controls is presented in Table 1. The

TABLE 1 Plasma-free amino acid concentrations in dogs with HCC, benign liver diseases, and healthy age-matched control dogs

Amino acid (nmol/mL)	Healthy controls, median (range) or mean (SD)	Benign liver diseases, median (range) or mean (SD)	HCC, median (range) or mean (SD)
Alanine	476.7 (353.6-599.9)	345.1 (222.6-467.5)	406 (270.1-541.9)
Arginine	104.1 (74.6-155.6)	86.9 (45.5-125.9)	80.8 (33.3-165.3)
Asparagine	51.2 (37.2-65.2)	41.6 (32.3-48)	53.7 (37.8-69.7)
Citrulline	54.4 (35.3-127.3)	67.8 (46.6-99.3)	67.9 (22.4-322.3)
Glutamic acid[a]	22.4 (15.1-42.6)	38.4 (27.1-50.7)[b]	48.1 (30.4-113.4)[b]
Glutamine	687.7 (557-818.4)	739.7 (629.3-850.2)	844.3 (604.5-1084.1)
Glycine	207 (145-269.1)	150.9 (112.7-189.2)	192.1 (128.1-256.1)
Histidine	80.3 (68-93.4)	69.9 (60.7-99.3)	77.5 (59.2-120.3)
Isoleucine	58.4 (37.4-77.1)	55.3 (49.9-208)	61 (35.4-109.7)
Leucine	115.9 (84-187.5)	114.1 (104.2-331.6)	126.2 (65.8-207.5)
Lysine	154.8 (87.6-254.2)	150.3 (105.8-185.1)	182.4 (92.1-381.7)
Methionine	62.6 (51.3-74)	50 (38.2-61.9)	58.3 (41.2-75.3)
Ornithine	15.5 (7.3-31.2)	18.8 (10.6-20.2)	20.6 (9.8-66.4)
Phenylalanine	62.4 (51.1-73.6)	65.3 (55.6-75)	63.1 (51.5-74.6)
Proline	170.9 (70.5-271.4)	93 (73.7-112.3)	125.7 (81.2-170.2)
Serine	124.7 (59.1-209.6)	96.5 (72.5-125.4)	107.1 (68.3-298.1)
Taurine	120.5 (79.1-161.9)	111 (50.7-171.3)	138 (64.3-211.7)
Threonine	194.6 (149.4-239.9)	153.6 (128.7-178.5)	174.1 (116-232.2)
Tryptophan	75.9 (37.1-94.8)	49.9 (27.5-108.8)	64.4 (35.4-154.2)
Tyrosine	47.8 (32.9-62.8)	42.3 (31.3-53.4)	39.9 (28.1-51.7)
Valine	180.7 (115.2-230)	168.2 (146.2-399.2)	190.6 (117.4-307.8)

Abbreviation: HCC, hepatocellular carcinoma.
[a]*P* values of <.0024 were statistically significant among groups after Bonferroni correction.
[b]*P* values of <.05 were statistically significant from control group value by post hoc analysis.

TABLE 2 Concentrations (nmol/mL) of plasma TAAs, BCAAs, and AAAs, as well as liver function indicators in dogs with HCC, benign liver diseases, and controls

Variable	Healthy controls, median (range) or mean (SD)	Benign liver diseases, median (range) or mean (SD)	HCC, median (range) or mean (SD)
TAAs	3116.2 (2623.4-3608.9)	2801.5 (2587.6-3015.4)	3174.5 (2644.3-3704.7)
BCAAs	349.8 (236.6-476.4)	335.3 (301-938.9)	378.9 (219.3-602)
AAAs	110.2 (86.8-133.6)	107.6 (88.2-127)	103 (82.2-123.7)
Fischer's ratio	3.4 (2.7-4.4)	3.5 (2.4-9.3)	4 (1.7-5.3)
BTR	7.8 (5.7-11.3)	9.4 (5.7-25.5)	10.2 (4.9-16.6)

Abbreviations: AAAs, aromatic amino acids; BCAAs, branched-chain amino acids; BTR, branched-chain amino acids to tyrosine ratio; TAAs, total amino acids.

concentrations of a single plasma amino acid (glutamic acid) were significantly different among the dogs with benign liver disease, HCC, and the control dogs (Table 1). Specifically, the plasma concentrations of glutamic acid were significantly higher in dogs with benign and malignant liver lesions than in the control group ($P < .0001$). However, no significant difference in PFAA profiles were identified between dogs with HCC and those with benign liver lesions ($P > .05$).

Correlation analysis identified no significant correlations among PFAA concentrations, serum liver enzyme activities, and lesion size in dogs with HCC ($P > .05$).

The concentrations of TAAs, BCAAs, and AAAs and the results of liver function indicators, including Fischer's ratio and BTR, were not significantly different among the 3 groups (all, $P > .05$). The concentrations of all PFAA subgroups and liver function indicators of dogs with HCC, benign liver disease, and controls are summarized in Table 2.

3.5 | PFAA concentrations after surgical treatment

Postoperative plasma samples for amino acid analysis were obtained from only 9 of the 26 dogs diagnosed with HCC. Plasma glutamic acid

concentrations 3 to 6 months after surgical resection for HCC were not significantly different from those obtained before surgical resection ($P > .05$).

4 | DISCUSSION

We investigated the utility of changes in PFAA profiles as diagnostic biomarkers for HCC in dogs. Although we determined that plasma glutamic acid concentrations were significantly higher in dogs with HCC compared to healthy dogs, no significant differences were found between the PFAA profiles of HCC dogs and those with benign liver disease. In addition, plasma PFAA concentrations were not correlated with liver enzyme activities or lesion size in dogs with HCC.

Metabolites, including amino acids, can be altered in both neoplastic tissues[23,24] and plasma.[7,10-16,18] Alterations in amino acid profiles and protein metabolism can reflect the effect of pathogenic changes because tumor development and growth require amino acids for protein synthesis, and both local and circulating amino acids are critical sources of metabolic fuel, leading to an increase in the mass of the affected tissue.[11,23]

Previous studies in humans have suggested that some amino acids and liver function indicators, including Fischer's ratio and BTR, are associated with HCC metabolism.[15,25-30] However, amino acid profiles are different between humans and dogs suffering from HCC, possibly because of differences in the causes and pathogenesis of liver neoplasms in humans and dogs. In our study, glutamic acid concentrations were significantly higher in both HCC dogs and dogs with benign liver lesions as compared to healthy control dogs. This result is consistent with the results of previous studies in dogs with lymphoma,[17] skin tumors,[23] and perianal tumors[31] and with those of certain studies of HCC in humans.[15,30] This result may be a consequence of increased gluconeogenesis in hepatic cells of dogs with focal liver lesions because of an increased rate of glucose utilization by cancer cells and nonmalignant proliferating cells—a typical signature of cancer that also occurs in both benign and early stage lesions, which is called the Warburg effect.[32] Moreover, this finding is in agreement with that of a previous report in humans showing increased concentrations of glucogenic amino acids, including glutamic acid, in HCC tissues.[33] However, no association was found between glutamic acid concentrations and tumor stage, although increased concentrations of glutamic acid were found in patients with cancer.[34]

Our study did not find significant differences between the PFAA profiles of HCC dogs and dogs with benign liver disease. However, because of the small number of dogs with benign lesions that underwent surgical resection, our data are inconclusive as to whether there is a nonspecific PFAA profile for either HCC or malignant liver tumors in dogs. In addition, there have been no reports regarding differences in PFAA profiles between benign changes and HCC in either human or veterinary medicine. Thus, further study is needed to determine whether PFAA profiles of HCC can distinguish this condition from benign structural changes.

Fischer's ratio and the BTR, which are indicators of liver function, were not decreased in dogs with HCC compared to healthy control dogs. These findings differed from those of previous studies in humans with HCC, in whom decreases in Fischer's ratio and the BTR have been reported. These results suggested that HCC in dogs does not affect liver function, in a similar fashion as in humans with HCC. This variation likely is a consequence of differences in the causes of HCC in humans and liver neoplasms in dogs. Specifically, the majority of HCC in humans are related to viral hepatitis and cirrhosis,[35] both of which lead to liver dysfunction.

To assess changes in protein metabolism after liver tumor resection, we investigated PFAA profiles 3 to 6 months after surgical treatment. We chose these time points because evidence in humans indicates that quality of life after liver resection returns to baseline at 3 months, with progressive and sustained improvements at 6 months.[36,37] Therefore, the period between 3 and 6 months after surgery may be a reasonable time point for assessing improvements in PFAA concentrations after surgery. However, we did not find significant improvement in plasma glutamic acid concentrations after surgical resection. This negative result may be a consequence of the fact that only a small number of dogs (9 of 26 dogs with HCC) returned for postsurgical follow-up evaluation during the study period. Thus, because of the small number of postsurgical follow-up evaluations, we cannot conclude whether changes in protein metabolism in HCC dogs return to normal after liver mass resection.

Our study had several limitations. First, the number of dogs with HCC and benign liver diseases that were surgically treated was small, as was the number of healthy age-matched controls. This fact may have resulted in an underpowered study for identifying changes in PFAA concentrations, especially when comparing HCC and benign liver diseases. Second, because of the low level of client follow-up at our referral hospital, only a small number of dogs with HCC were included in the postoperative group. This fact may have affected the results of the postsurgical amino acid analyses. However, we confirmed that our included dogs with HCC that were returned follow-up did not have any comorbidities on the date of the postoperative blood collection that could have confounded the PFAA results. Thus, further study is needed to investigate whether any form of hepatic surgery results in improved postoperative PFAA concentrations. Next, no published reference ranges of PFAA concentrations are available, but plasma was selected for amino acid concentration measurement because similar studies have been performed in humans with HCC[15,26,27] and in dogs with various types of tumors.[10,16,18] Therefore, to minimize this limitation, a control group of healthy age-matched dogs was used for comparison instead of reference concentration ranges. In addition, we did not use breed-matched control groups for comparison to dogs with HCC because of the small number of healthy control dogs examined at our referral veterinary teaching hospital. Thus, although no reports have indicated breed variations in PFAA concentrations, additional studies using breed-matched controls should be conducted to eliminate any potential confounding effects.

In conclusion, we found that increased plasma glutamic acid concentrations may involve or be a consequence of a liver mass lesion.

However, PFAA profiles of dogs with HCC could not be distinguished from those of dogs with benign liver diseases. In addition, postoperative glutamic acid concentrations of dogs with HCC did not improve after surgical resection compared to preoperative concentrations. These results indicate that PFAA profiles may not be specific diagnostic biomarkers for detecting HCC in dogs. Additional studies with a larger population of dogs should be conducted to confirm our results.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All animal procedures were approved by the Hokkaido University Animal Care and Use Committee.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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