FULL PAPER *Biochemistry*

Profiling of Serum Metabolites in Canine Lymphoma Using Gas Chromatography Mass Spectrometry

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ABSTRACT. Canine lymphoma is a common cancer that has high rates of complete remission with combination chemotherapy. However, the duration of remission varies based on multiple factors, and there is a need to develop a method for early detection of recurrence. In this study, we compared the metabolites profiles in serum from 21 dogs with lymphoma and 13 healthy dogs using gas chromatography mass spectrometry (GC-MS). The lymphoma group was separated from the control group in an orthogonal projection to latent structure with discriminant analysis (OPLS-DA) plot using ions of m/z 100–600, indicating that the metabolites profiles in lymphoma cases differed from those in healthy dogs. The lymphoma group was also separated from the control group on OPLS-DA plot using 29 metabolites identified in all serum samples. Significant differences were found for 16 of these metabolites with higher levels in the lymphoma group for 15 of the metabolites and lower levels for inositol. An OPLS-DA plot showed separation of the lymphoma and healthy groups using these 16 metabolites only. These results indicate that metabolites profile with GC-MS may be a useful tool for detection of potential biomarker and diagnosis of canine lymphoma.

KEY WORDS: GC-MS, lymphoma, metabolomics

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Canine lymphoma is a common cancer that occurs in solid organs. This disease constitutes up to 24% of all malignant canine tumors and is the most common canine hematopoietic cancer [11, 28, 29]. Initial diagnosis of canine lymphoma is made by physical examination, complete blood count, serum biochemistry profile, urinalysis and histological evaluation, while the disease is classified based on anatomic location, histologic criteria and immunophenotype characteristics [28]. Systemic chemotherapy continues to be the therapy of choice. Conventional chemotherapy induces complete remission in approximately 60 to 90% of cases, and median survival times are 6 to 12 months [27] with response rates and duration varying based on disease location, histological grade and immunophenotype [28]. Serum lactate dehydrogenase activity may be predictive of recurrence of lymphoma in dogs, but the sensitivity is low [16]. Therefore, a new method is required for early detection of recurrence.

"Omics" disciplines including genomics, transcriptomics, proteomics and metabolomics have the goal of analysis of the components of a living organism entirely [5]. Integration of these techniques is the study researching interrelationships of several or all the elements in a biological system [24]. Metabolomics can be defined as the non-targeted analysis of all small molecule metabolites (≤1,500 Da) produced by the body [1]. Metabolites participate in general metabolic reactions and are required for maintenance, growth and normal function of cells [2]. These metabolites are present in biofluids, such as urine and blood, and can be collected relatively easily. Metabolomics also has advantages over other 'omics', because the metabolic network is downstream from

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Table 1. Characteristics of healthy dogs and dogs with lymphoma

	No.	Breed	Age (y)	Gender	Region	Cell type
Control	1	Toy poodle	3	Cast	-	-
	2	Mongrel	5	Female	-	-
	3	Miniature schnauzer	1	Cast	-	-
	4	Pomeranian	7	Male	-	-
	5	Shih tzu	3	Spay	-	-
	6	Toy poodle	6	Cast	-	-
	7	Mongrel	11	Spay	-	-
	8	Labrador retriever	5	Spay	-	-
	9	Labrador retriever	2	Spay	-	-
	10	Papillon	2	Spay	-	-
	11	Borzoi	3	Spay	-	-
	12	Miniature dachshund	13	Cast	-	-
	13	Golden retriever	2	Spay	-	-
Lymphoma	1	Chihuahua	4	Male	M	B
	2	Flat-coated retriever	8	Male	GI	T
	3	Golden retriever	11	Spay	GI	N.I.
	4	Beagle	6	Male	GI	T
	5	Mongrel	10	Male	M	B
	6	Pug	4	Male	GI	T
	7	American cocker spaniel	4	Male	M	B
	8	Welsh corgi	8	Cast	M	B
	9	Welsh corgi	8	Spay	M	N.I.
	10	Toy poodle	5	Spay	M	B
	11	Labrador retriever	9	Cast	M	N.I.
	12	Mongrel	17	Spay	Spleen	N.I.
	13	Welsh corgi	8	Spay	M	N.I.
	14	Beagle	9	Cast	M	B
	15	Golden retriever	9	Spay	M	N.I.
	16	Mongrel	16	Male	M	B
	17	Yorkshire terrier	9	Female	M	B
	18	Golden retriever	3	Cast	M	B
	19	Boston terrier	8	Female	GI	T
	20	Shiba	9	Spay	GI	T
	21	Shiba	11	Cast	GI	B

Age is shown in years. 'Spay' indicates a spayed female, and 'Cast' indicates a castrated male. M: Multicentric lymphoma, GI: Gastrointestinal lymphoma, N.I.: Not investigated.

gene expression and protein synthesis and thus more closely reflects the activities of the cell at a functional level [8]. Gas chromatography mass spectrometry (GC-MS) can be used to analyze volatile substance in serum, such as amino acids, organic acids and fatty acids, while GC-MS screening of urine can provide evidence for molecular diagnosis of inborn errors of metabolism, such as methylmalomic aciduria [14]. Serum metabolomic analysis has also been shown to be useful to identify potential biomarkers of colorectal cancer and gastric cancer [19, 25]. In this study, we used GC-MS to examine metabolites profiles in serum from dogs with lymphoma with the goal of identifying biomarkers for diagnosis of canine lymphoma.

MATERIALS AND METHODS

Materials: Serum samples from 13 healthy dogs and 21 dogs with lymphoma (Table 1) were obtained from the Veterinary Medical Center of Osaka Prefecture University and from animal hospitals in Osaka and Hyogo Prefectures. The samples were stored at −30°C until use.

GC-MS analysis: Twenty-five μl of serum were mixed with 5 μl of 1 mg/m*l* 2-isopropylmalic acid (Sigma-Aldrich, Tokyo, Japan) as an internal standard (IS), and 900 μl of 70% ethanol were added. The mixture was voltexed and centrifuged at 15,000 rpm for 5 min at 4°C, and then, 900 μl of the supernatant were transferred to a clean vial and dried under a N_2 gas stream. After drying, 25 μl of *N,O*-bis (trimethylsilyl) trifluoroacetamide with 10% trimethylchlorosilane (Thermo Scientific, Tokyo, Japan) were added to the vial, and the mixture was incubated for 30 min at 90°C. The solution was transferred to a clean vial and analyzed by GC-MS. GC-MS analysis was performed using a GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) with a DB-5 column (Length: 30 m, Film: 1.00 μm and Inside diameter: 0.25 mm, Agilent Technologies, Santa Clara, CA, U.S.A.). The flow

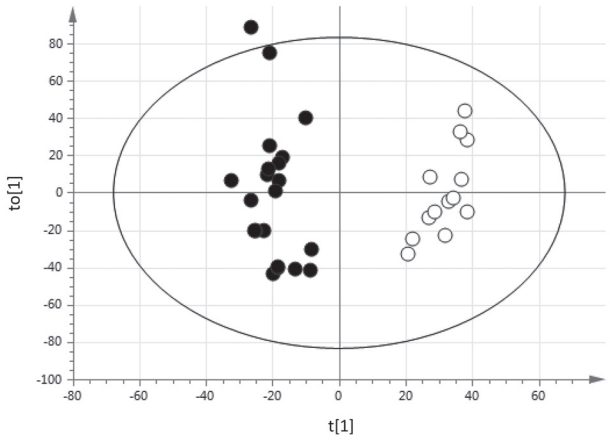

Fig. 1. OPLS-DA score plot using all ions with m/z 100–600 for healthy control dogs (white circles, n=13) and dogs with lymphoma (black circles, n=21).

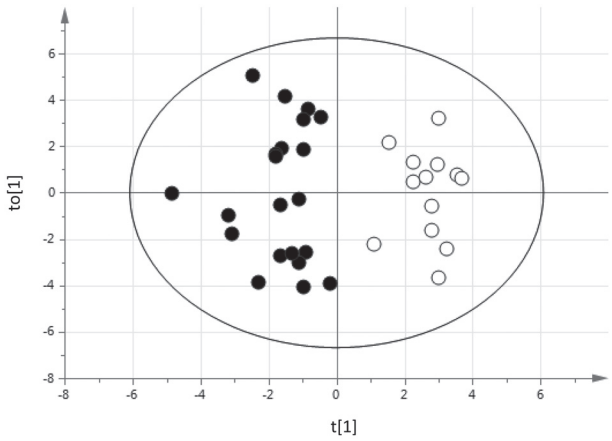

Fig. 2. OPLS-DA score plot using the 29 metabolites identified in serum from healthy control dogs (white circles, m=13) and dogs with lymphoma (black circles, n=21).

rate was 24.5 m*l*/min. The GC column temperature was programmed to rise from 100 to 300°C at a rate of 4°C per min. The sample was injected in splitless mode. The ion source temperature was 230°C. Chromatogram acquisition, detection of peaks and data processing were performed using Shimadzu GCMS solution software ver. 2.71 (Shimadzu). Low molecular weight metabolites were identified using the NIST library (NIST08) with peaks assigned based on a similarity index >75. In addition to the automatic assessment, the identified metabolites were confirmed manually. The peak area of each metabolite was divided by that of the IS to give a relative value. The serum levels of metabolites were shown as fold ratio. The relative value of the dogs with lymphoma was compared with the mean of the healthy dogs.

Statistical analysis: Multivariate analysis was performed using orthogonal projection to latent structure with discriminant analysis (OPLS-DA) in SIMCA 13.0 software (Umetrics, Umea, Sweden). Statistical significance was analyzed using a Mann-Whitney U test.

RESULTS

Serum samples from 21 dogs with lymphoma were subjected to GC-MS analysis. Data for ion peaks at m/z 100–600 were converted into relative values and analyzed with OPLS-DA (Fig. 1). The lymphoma group was separated from the control group on the OPLS-DA score plot. R^2X (cum), R^2Y (cum) and Q^2 (cum) were 0.369, 0.947 and 0.76, respectively.

A total of 53 metabolites were identified using the NIST library, including 29 metabolites that were detected in all samples. OPLS-DA for the 29 metabolites also showed separation of the control and lymphoma groups (Fig. 2). R^2X (cum), R^2Y (cum) and Q^2 (cum) were 0.611, 0.837 and 0.69, respectively.

The relative levels of the 29 metabolites detected by GC-MS in the serum of the lymphoma cases were compared with those in control dogs. A total of 16 metabolites had significantly different levels ($P<0.05$), including 13 with $P<0.01$ (Fig. 3). Fifteen of these metabolites had higher levels in the lymphoma group, while inositol had lower levels in the lymphoma group.

OPLS-DA for only these 16 metabolites also showed separation of the control and lymphoma groups (Fig. 4). R^2X (cum), R^2 Y (cum) and Q^2 (cum) were 0.545, 0.845 and 0.58, respectively. Sera from 3 cases diagnosed as complete remission after the treatment according to the chemotherapy protocol of University of Wisconsin-Madison [6] were also analyzed with GC-MS, and the cases were plotted on the control group side on an OPLS-DA score plot by an OPLS discriminant model using the 16 metabolites significantly differed between the lymphoma and control group (Fig. 4).

DISCUSSION

Canine lymphoma has high complete remission rates, but short and variable duration of remission after treatment [28]. In this study, we analyzed small molecules in serum using GC-MS to examine differences of metabolites profiles between healthy dogs and lymphoma cases with the goal of identifying potential biomarkers for diagnosis of canine lymphoma.

The R^2X (cum) and R^2Y (cum) are cumulative fraction of sum of squares of the entire X and all y-variables explained by the extracted components. The Q^2 (cum) is the cumulative Q^2 for all the x-variables and y-variables for the extracted components. Values of R^2Y (cum) and Q^2 (cum) close to 1.0 indicate an excellent model. The result showed a difference of metabolite composition between the groups using OPLS-DA based on all ions with m/z 100–600 detected in serum (Fig. 1). The serum profiles clearly differed in lymphoma cases compared to control on OPLS-DA score plot using 29 metabolites detected in all cases, although R^2Y (cum) and Q^2 (cum) were decreased (Fig. 2). These results indicate

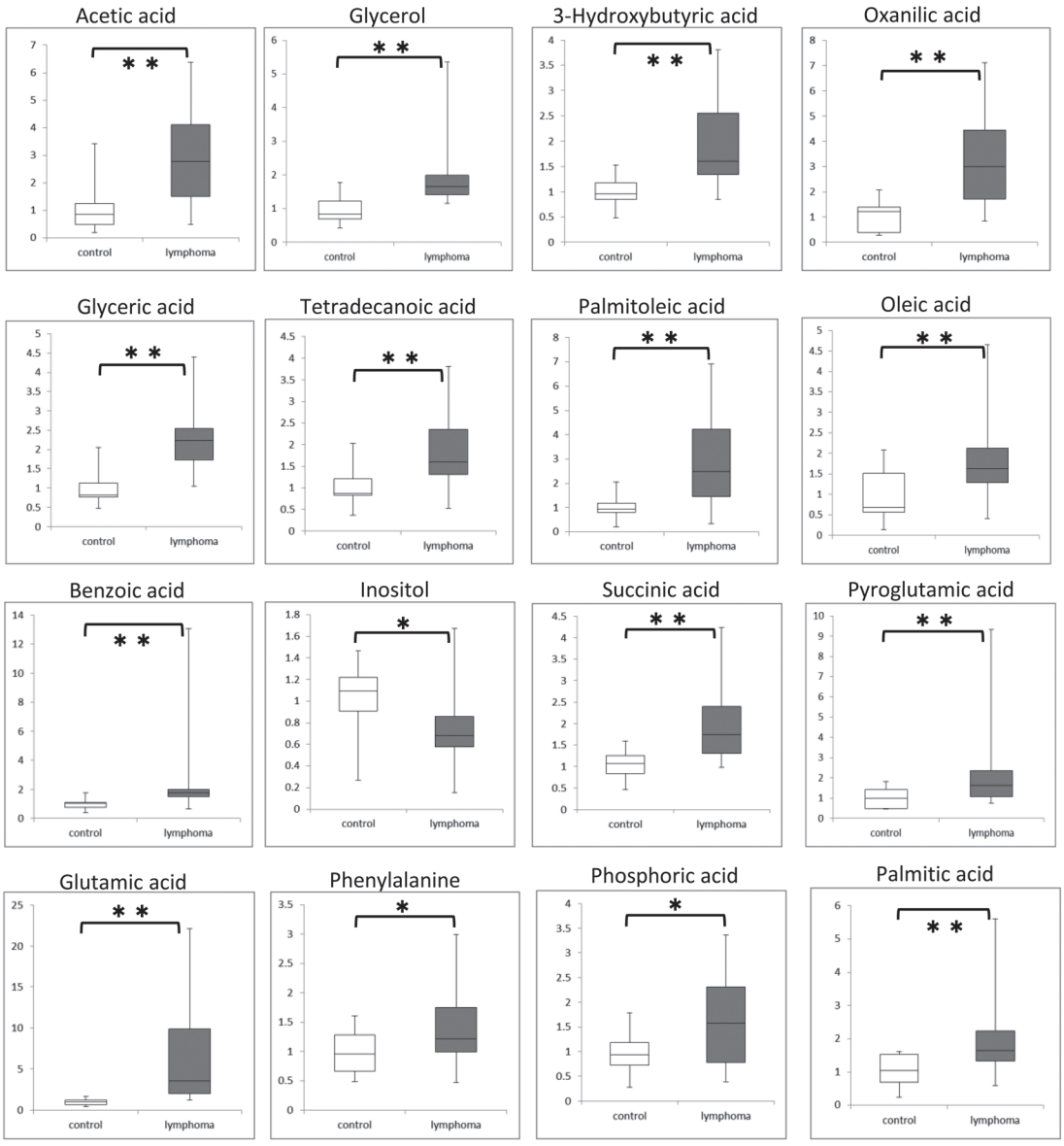

Fig. 3. The levels of serum metabolites significantly different between the healthy control and lymphoma groups. The ends of the whisker indicate the minimum and maximum. Asterisks indicate a significant difference by Mann Whitney U test (**P*<0.05; *P*<0.01).

that the serum metabolites profiles of lymphoma dogs differs from those of healthy dogs and that it is possible to discriminate dogs with lymphoma from healthy dogs by analyzing serum. The lymphoma cases analyzed were composed of various anatomic location and/or histological criteria in this study. Further studies are required to evaluate the differences between lymphoma characteristics depending on its location and histological criteria.

A comparison of the levels of metabolites between the control and lymphoma groups showed significant differences for 16 metabolites of 29 metabolites (Fig. 3). A similar comparison of humans with pancreatic cancer and healthy volunteers showed significant changes in the levels of 18 of 60 detected metabolites [20]. Similarly, the levels of 18 of 44 endogenous metabolites were found to differ significantly between patients with gastric cancer and healthy volunteers [25]. Thus, our results and the method are in general agreements with these reports.

Of the 16 metabolites with different levels in the control and lymphoma groups, the levels of several fatty acids, including palmitoleic acid, oleic acid and palmitic acid, were increased in the lymphoma cases (Fig. 3). These molecules are essential for formation of phospholipid of lipid membranes and for energy metabolism. In animals, fatty acids for

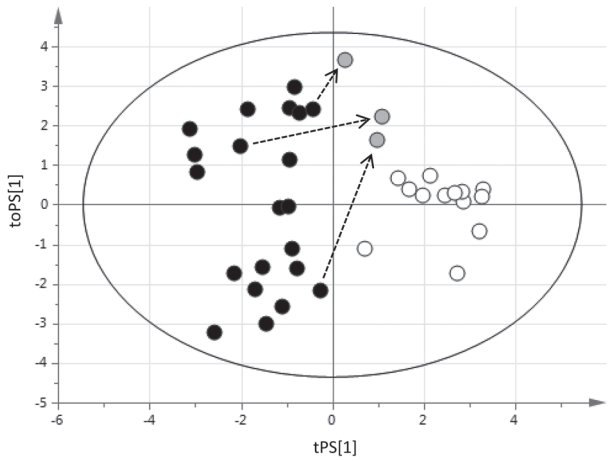

Fig. 4. OPLS-DA score plot made by a discriminant model with the 16 metabolites. Controls were indicated as white circles (n=13), dogs with lymphoma were as black circles (n=21) and the complete remission cases were as gray circles (n=3). The movement of the plot showed with the arrow.

metabolism are derived from dietary sources and from endogenous synthesis catalyzed by fatty acid synthase (FASN) [17]. Tumor cells depend upon endogenous fatty acid synthesis for more than 93% of triacylglycerol fatty acids, which is higher than in normal cells [17]. FASN primarily produces palmitic acid, which is a 16-carbon saturated fatty acid [13], and FASN is highly expressed in most malignant tumors [17], including breast cancer [10], prostate cancer [18], colorectal cancer [30], T cell lymphoma [12] and mantle cell lymphoma [7] in human. The levels of palmitic acid and oleic acid are significantly elevated, and those of palmitoleic acid in serum phospholipids are significantly decreased in humans with non-Hodgkin lymphoma [4], while inhibition of FASN activity reduces proliferation and promotes apoptosis in a mouse melanoma model [3]. Our findings are consistent with these results for the effects and changes of palmitic acid and oleic acid in cancer, but not consistent with changes of palmitoleic acid. These findings suggest that FASN expression might have an important function in canine lymphoma and changes of fatty acid levels might be different between human and canine. It is necessary to study lipid metabolism in dog with malignant tumor.

The level of phenylalanine and glutamic acid increased in serum of dogs with lymphoma group (Fig. 3). The plasma phenylalanine level has been shown to increase in human non-small-cell lung cancer [15], but decreases in human esophageal cancer, endometrial cancer, colorectal cancer and breast cancer [9, 21, 22]. The plasma glutamic acid level has been shown to increase in human colorectal cancer and breast cancer [22]. These changes in plasma levels may make phenylalanine and glutamic acid useful as potential biomarkers for early detection of several types of cancer. The mechanisms of the amino acid changes in serum from tumor patient remained unclear.

The levels of 3-hydroxybutyric acid, palmitoleic acid, glutamic acid and glyceric acid increased in the lymphoma group. In serum from cases of human pancreatic cancer at different stages, 3-hydroxybutyric acid tends to increase in Stage IVb, palmitoleic acid increases in Stage III and Stage IVa, glutamic acid increases in Stage III and Stage IVb, and glyceric acid decreases in Stages IVa and IVb, indicating that these metabolites reflect the stage [20]. Our results are in agreement with these findings, except for glyceric acid. Further studies are required to determine the relationship between cancer stage and metabolite levels in serum.

The OPLS-DA score plot showed separation of the control and lymphoma groups using the 16 metabolites with significantly different levels between the two groups. This result indicates that these metabolites might be useful as potential biomarkers for canine lymphoma. It is reported that blood metabolites profiles of multiple myeloma and pediatric acute lymphoblastic leukemia patients in remission differ from those of the patients at diagnosis in human [23, 26]. Although the number of the cases was limited, the cases diagnosed as complete remission were plotted on the control group side on the OPLS-DA score plot by an OPLS discriminant model using the 16 metabolites significantly differed between the lymphoma and control groups, indicating that serum metabolites profiles of the complete remission cases are different from those of lymphoma dogs (Fig. 4). These suggested that there is possibility to diagnose remission and recurrence of canine lymphoma using serum metabolites profile. It is necessary to study to evaluate the serum metabolites of the cases diagnosed as complete remission with the increasing number of cases.

In conclusion, analysis of metabolites profile in serum using GC-MS is useful to find potential biomarkers for canine lymphoma, and the metabolites found as potential biomarkers also could be useful for diagnosing lymphoma. It is required to examine serum metabolites profile of malignant cancers other than lymphoma and various non-neoplastic diseases to establish a lymphoma specific diagnosing biomarker.

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