FULL PAPER *Clinical Pathology*

Retrospective Diagnosis of Feline GM2 Gangliosidosis Variant 0 (Sandhoff-Like Disease) in Japan: Possible Spread of the Mutant Allele in the Japanese Domestic Cat Population

Osamu YAMATO[1)]*, Daisuke HAYASHI[2)], Hiroyuki SATOH[2)], Toru SHODA[2)], Keiko UCHIDA[3)], Hiroyuki NAKAYAMA[4)], Hiroki SAKAI[5)], Toshiaki MASEGI[5)], Atsuko MURAI[5)], Tsuneyoshi IIDA[6)], Hiromi HISADA[7)], Atsunori HISADA[7)], Masahiro YAMASAKI[2)], Yoshimitsu MAEDE[2)] and Toshiro ARAI[8)]

[1)]Laboratory of Clinical Pathology, Department of Veterinary Clinical Sciences, Faculty of Agriculture, Kagoshima University, Kagoshima 890–0065, [2)]Laboratory of Internal Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, [3)]Ichikawabashi Hospital of Kariya Small Animal Hospital, Ichikawa-shi, Chiba 272–0034, [4)]Laboratory of Veterinary Pathology, Graduate School of Agriculture and Life Sciences, the University of Tokyo, Bunkyo-ku, Tokyo 113–8657, [5)]Laboratory of Veterinary Pathology, Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, Gifu 501–1193, [6)]Iida Animal Clinic, Kakamigahara-shi, Gifu 509–0132, [7)]Hisada Animal Hospital, Fushimi-ku, Kyoto 601–1374 and [8)]Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Nippon Veterinary and Life Science University, Musashino-shi, Tokyo 180–8602, Japan

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ABSTRACT. GM2 gangliosidosis variant 0 (human Sandhoff disease) is a lysosomal storage disease caused by simultaneous deficiencies of acid β-hexosaminidase (Hex) A and Hex B due to an abnormality of β-subunit, a common component in these enzyme molecules, which is coded by the *HEXB* gene. In the present study, a retrospective diagnosis was performed in 2 previous suspected cases of feline Sandhoff-like disease using a DNA test to detect the causative mutation identified previously in 4 cats in 2 other families of Japanese domestic cats. Enzymic analysis was also performed using stored leukocytes and plasma collected from the subject families in order to investigate the usefulness of enzymic diagnosis and genotyping of carriers. The DNA test suggested that the 2 cases were homozygous recessive for the mutation. Consequently, 6 cats homozygous for the same mutation have been found in 4 separate locations of Japan, suggesting that this mutant allele may be spread widely in the Japanese domestic cat populations. In enzymic analysis, Hex A and Hex B activities in leukocytes and plasma measured using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as a substrate were negligible in affected cats, compared with those in normal and carrier cats. However, there was a wide overlap in enzyme activity between normal and carrier cats. Therefore, it was concluded that enzymic analysis is useful for diagnosis of affected cats, but is not acceptable for genotyping of carriers.

KEY WORDS: acid β-hexosaminidase A, GM2 gangliosidosis variant 0, Japanese domestic cat, retrospective diagnosis, Sandhoff disease.

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GM2 gangliosidoses are lysosomal diseases caused by the excessive accumulation of GM2 ganglioside and related glycolipids in lysosomes, especially in the lysosomes of neurons [7]. These diseases are inherited as autosomal recessive traits. The major clinical signs are progressive motor and psychointellectual dysfunctions, startle response and blindness. The hydrolysis of GM2 ganglioside is catalyzed by lysosomal acid β-hexosaminidase (Hex), but this hydrolysis requires that the ganglioside be complexed with a substrate-specific cofactor, the GM2 activator protein. There are two main isoenzymes of Hex: Hex A, a heterodimer of $\alpha\beta$ subunits, and Hex B, a homodimetric structure $\beta\beta$, and only Hex A can act on the complex of GM2 ganglioside and GM2 activator protein. Defects in any of the three related genes may lead to GM2 gangliosidosis: *HEXA*, which encodes the α-subunit of Hex A; *HEXB*, which encodes the β-subunit of Hex A and Hex B; or *GM2A*, which encodes the GM2 activator protein. As a result, there are three main forms of GM2 gangliosidosis: Tay-Sachs disease (B variant), resulting from mutations of the *HEXA* gene, is associated with deficient activity of Hex A but normal Hex B; Sandhoff disease (0 variant), resulting from mutations of the *HEXB* gene, is associated with deficient activity of both Hex A and Hex B; and AB variant or GM2 activator deficiency disease caused by mutations of the *GM2A* gene, characterized by normal Hex A and Hex B but the inability to form a functional complex of GM2 ganglioside and GM2 activator protein, required for hydrolysis of GM2 ganglioside.

In animals, GM2 gangliosidosis has been reported in dogs [2, 4, 6, 16, 22], cats [3, 11, 13, 23], pigs [14, 15] and deer [5]. In cats, GM2 gangliosidosis corresponding to human Sandhoff disease has been identified in domestic shorthaired cats [3], Korat cats [13] and Japanese domestic cats [23], and GM2 activator protein deficiency corresponding to human AB variant was identified in a family of domestic cats [11]. Each of these diseases may have several different causative mutations. In the feline Sandhoff models, the mutations are as follows: an inversion of 25 base pairs at

* CORRESPONDENCE TO: YAMATO, O., Laboratory of Clinical Pathology, Department of Veterinary Clinical Sciences, Faculty of Agriculture, Kagoshima University, Kagoshima 089–0065, Japan.
e-mail: osam@agri.kagoshima-u.ac.jp

nucleotide position 1467–1491 in the open reading frame (ORF) of the feline *HEXB* gene in domestic shorthaired cats in the U.S.A. [10], a deletion of cytosine at position 39 in the ORF of this gene in Korat cats in the U.S.A. and 12 other countries [12], and a single nucleotide substitution from cytosine to thymine at position 667 (667C>T) in Japan [9]. The mutation of feline AB variant model has been identified as a deletion of the 4 base pairs around the 3' end of the feline *GM2A* cDNA in the U.S.A. [11].

In the present study, two previous cases (cases 1 and 2) of Japanese domestic cats with degenerative brain diseases characteristic of lysosomal storage diseases were diagnosed retrospectively using the molecular diagnostic method to detect the 667C>T found in Japanese domestic cat families. The retrospective diagnosis of these cases suggested that this mutant allele might be spread widely in the Japanese domestic cat populations. In addition, the activities of Hex A and Hex B were measured in leukocytes and plasma, which had been collected from cats in families with feline Sandhoff-like disease, to investigate the usefulness of enzymic analysis for diagnosis and genotyping of carriers in this feline disease.

MATERIALS AND METHODS

DNA mutation assay: The DNA mutation assay was performed according to the method reported previously [9] with a slight modification. Briefly, PCR was carried out in a 25-μl reaction mixture containing 1x Ampdirect-A (Ampdirect for Human Blood, Shimadzu Corp., Kyoto, Japan), 0.6 units of Taq polymerase (Takara EX Taq, Takara Shuzo, Tokyo, Japan), 0.2 mM deoxynucleoside 5'-triphosphate, a DNA template and 12.5 pmol of primers (forward: 5'-GTC CAT ACG GTG ATT GAA TAT GCC AGA GTA-3' and reverse: 5'-CTT TTC CCC AAG ACT GTG TAT GTC-3'). After the initial denaturation at 95°C for 2 min, 40 cycles of amplification were carried out, at a denaturing temperature of 95°C for 30 sec, an annealing temperature of 55°C for 30 sec, and an extension temperature of 72°C for 1 min. Extension during the last cycle was carried out at 72°C for 5 min. The PCR products were digested with *Rsa*I (Nippon Gene, Tokyo, Japan) at 37°C for 90 min in a 10-μl reaction mixture containing 8 μl PCR products, 1 μl 10x reaction buffer attached by the manufacturer, and 5 units of *Rsa*I. The fragments were subjected to electrophoresis in 3% (wt/vol) agarose gel (Agarose 21, Nippon Gene) buffered with Tris-acetate-EDTA solution. The gels were stained with ethidium bromide and visualized under a UV transilluminator. As a template for PCR, blood smear specimens in case 1, paraffin-embedded liver in case 2, and whole blood and liver homogenate in control cats were prepared according to the method for a direct PCR reported previously [20]. Dried powdered blood was stripped from the blood smear specimens of case 1 using an 18-gauge needle, then put into an empty PCR tube and spun down on the tube bottom, and the PCR reaction mixture was layered over the specimen without mixing just prior to starting PCR. A fine chip of liver tissue was obtained from the paraffin-embedded block of case 2 using an 18-gauge needle, then washed in xylene to remove paraffin and suspended in an appropriate amount of pure water. One or 2 μl of this suspension as well as whole blood and liver homogenate was added to the reaction mixture without mixing just prior to starting PCR.

Enzyme assay: Leukocyte pellets and plasma that had been prepared and stored at –80°C for several months were used in the present study. These samples were mainly obtained from cats belonging to a family with Sandhoff-like disease in Adachi ward, Tokyo (mark A in Fig. 1), which was reported previously as the first identification of this disease in Japanese domestic cats [23]. Genotyping was carried out using the *HEXB* mutation assay described above. In this family, there were two affected cats, 4 heterozygous carriers and 7 normal cats [9]. Of the 7 normal cats, 4 cats were used for this enzymic analysis. Blood was also collected from an affected cat, which was 1 of 2 stray littermate cats in Musashino city, Tokyo (mark B in Fig. 1), also reported previously [8], and from 2 genotypically normal Japanese domestic cats unrelated to these families. Plasma samples were prepared from heparinized blood by centrifugation. Leukocytes were isolated by a differential sedimentation procedure using 3% dextran (Wako Pure Chemical Industries, Osaka, Japan) in physiological saline [18]. The activities of total Hex and heat-stable Hex B were measured spectrofluorometrically, using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma Co., St. Louis, MO, U.S.A.) as an artificial substrate according to the method

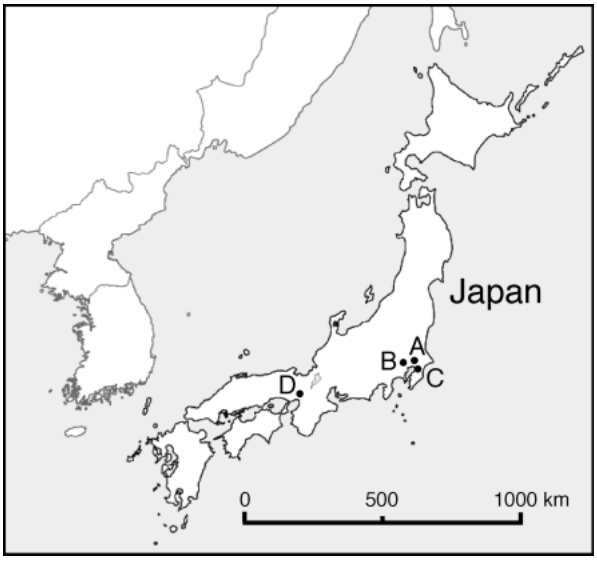

Fig. 1. Map of Japan and neighboring countries, indicating marks A to D where affected cats were found. Mark A: Adachi ward, Tokyo; the first Japanese domestic cat family with Sandhoff-like disease was identified in 2002. Mark B: Musashino city, Tokyo; two affected littermate kittens were found in 2002. Mark C: Ichikawa city, Chiba prefectur; case 1 was found in 1994. Mark D: Toyonaka city, Osaka prefecture; case 2 was found in 2004.

described previously [24]. The activity of Hex A was calculated by determining the difference between these.

Statistical analysis: Statistical analysis was performed by a 1-way factorial ANOVA with post hoc tests (Tukey method). Values of $P<0.05$ were considered significant.

RESULTS

Case 1: In 1994, a 3-month-old female kitten of Japanese domestic cat with a few weeks history of ataxia and head tremor was presented to a private veterinary hospital in Ichikawa city in Chiba prefecture, Japan (mark C in Fig. 1). This kitten was the progeny of littermate parents, according to the owner. Case 1 weighed 900 g and appeared dwarfed. Hematological examinations demonstrated leukocytosis (21,700 /μl) due to lymphocytosis (8,100 /μl) and neutrophilia (13,100 /μl), with cytoplasmic vacuolation in some lymphocytes. Neurological signs progressed gradually, with generalized muscular atrophy and blindness observed at 7 months of age. Finally case 1 became lethargic and died at 8 months of age. A lysosomal storage disease was suspected in this kitten, because intracytoplasmic inclusions and vacuoles were observed in neurons (data not shown). However, definitive diagnosis using biochemical or molecular analysis had not been established, and only blood smear specimens were available as a DNA sample for a retrospective molecular diagnosis.

Case 2: This case was a stray male kitten with an inability to eat well due to motor dysfunction in Toyonaka city, Osaka prefecture in 2004 (mark D in Fig. 1), and was brought to a regional private hospital with dysphagia and motor disability. Case 2 weighed 760 g and appeared dwarfed, although its true age was not known. Hematological examinations demonstrated leukocytosis (26,800–41,600 /μl), but further hematological examination was not performed to determine its leukogram and/or morphological abnormalities including lymphocyte vacuolation. The neurological symptoms progressed, and case 2 died two months after being first examined. Histopathological examination demonstrated intracytoplasmic inclusions and vacuoles in swollen neurons throughout the CNS (data not shown), suggesting a lysosomal storage disease. One year after the death of case 2, ganglioside composition was determined in formalin-fixed brain samples from case 2, using high performance thin-layer chromatography [23] resulting in the diagnosis of GM2 gangliosidosis due to marked accumulation of GM2 ganglioside in the brain (Table 1). The variant of GM2 gangliosidosis could not be determined, because enzymic analysis is not available for formalin-fixed tissue samples. Paraffin-embedded liver was used as a DNA sample for a retrospective molecular diagnosis two years after the death of case 2.

Molecular diagnosis: The result of the mutation assay for the 667C>T mutation is shown in Fig. 2. This assay suggested that case 1 (lane 7) and case 2 (lane 6) are homozygous recessive for the mutation, because a 91-bp DNA fragment amplified by PCR could not be digested with an endonuclease *Rsa*I as in an affected control cat (lane 3) and two affected littermate cats (lanes 4 and 5) reported previously [8, 9].

Table 1. Ganglioside composition of brain from an affected cat (case 2) and a control cat

Gangliosides*	Case 2	Control
Total	2947	641
GM2	1771	ND
GM1	117	92
GD1a	312	284
GD1b	102	94
GT1b	195	171

* Values are expressed as nmol of *N*-acetylneuraminic acid per gram wet weight of formalin-fixed cerebrum in case 2 and fresh frozen cerebrum in the control cat which died shortly after birth as a result of dystocia.
ND: Not detected.

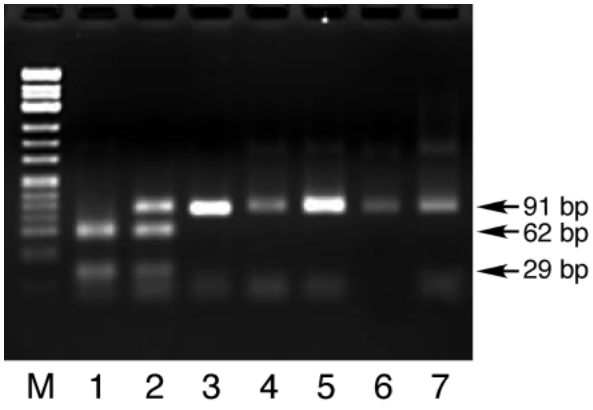

Fig. 2. Genotyping of Japanese domestic cats suspected of Sandhoff-like disease by DNA mutation assay using direct PCR and restriction endonuclease digestion. Lane M: marker, lanes 1–3: controls (1: normal, 2: carrier, 3: affected) from the first identified family in Adachi ward, Tokyo, lanes 4–5: two affected littermate kittens in Musashino city, Tokyo, lane 6: case 2 in Toyonaka city, Osaka prefecture, and lane 7: case 1 in Ichikawa city, Chiba prefecture. As a template for the direct PCR, whole blood in lanes 1–3 and 5, liver homogenate in lane 4, suspension of liver chip from paraffin-embedded block in lane 6, and dried powdered of blood from blood smear specimens in lane 7 were used.

Enzymic analysis: Hex A and B activities in leukocytes are shown in Fig. 3. The leukocyte Hex A activity in affected cats (1.2 ± 1.6 nmol/hr/mg protein, mean ± standard deviation) was markedly lower than the activity in normal and carrier cats, and significantly ($P<0.01$) lower than that in normal cats (1061 ± 449 nmol/hr/mg protein). The activity in carriers (506 ± 318 nmol/hr/mg protein) was approximately half of the normal mean activity. However, there was an overlap in enzyme activity, and therefore there was no significant difference between normal and carrier cats. The leukocyte Hex B activity in normal, carrier and affected cats was 236 ± 122, 101 ± 51 nmol/hr/mg protein and below

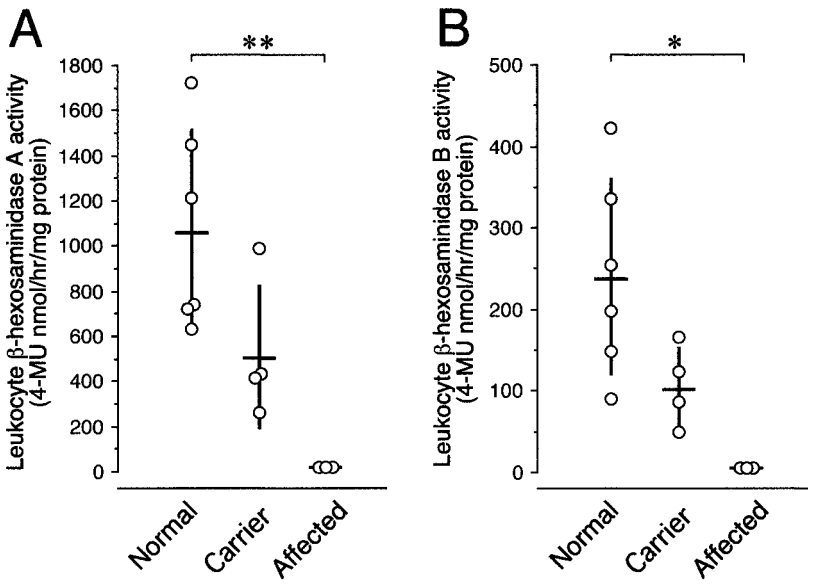

Fig. 3. (A) Leukocyte *β*-hexosaminidase A activity and (B) leukocyte *β*-hexosaminidase B activity measured with 4-methylumbelliferyl *N*-acetyl-*β*-D-glucosaminide (4-MU) in normal, carrier, and affected cats. Horizontal bars represent mean values. Vertical bars represent standard deviations. * $P<0.05$ and ** $P<0.01$ by 1-way factorial ANOVA with post hoc tests.

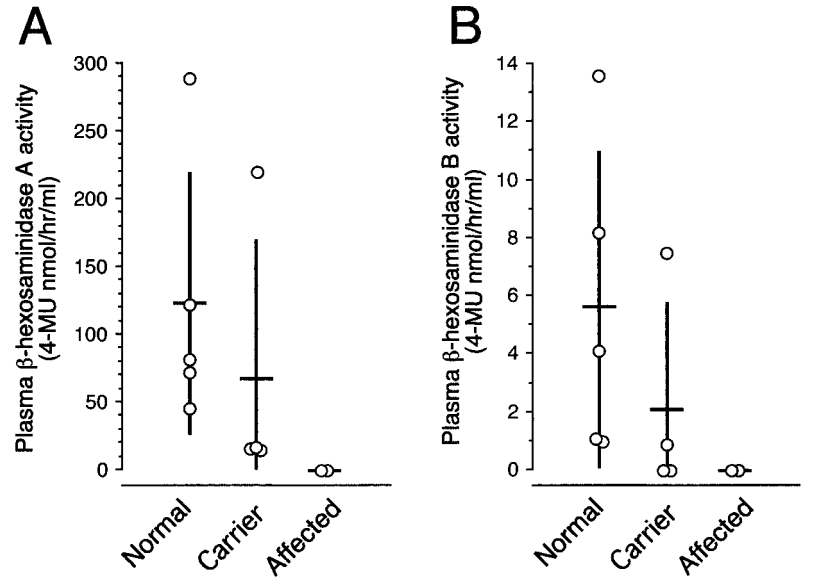

Fig. 4. (A) Plasma *β*-hexosaminidase A activity and (B) plasma *β*-hexosaminidase B activity measured with 4-methylumbelliferyl *N*-acetyl-*β*-D-glucosaminide (4-MU) in normal, carrier, and affected cats. Horizontal bars represent mean values. Vertical bars represent standard deviations.

detection limit, respectively (Fig. 3B). The difference in leukocyte Hex B activity in the three genotypes was similar to that in Hex A activity. The Hex B activity in affected cats was significantly ($P<0.05$) lower than that in normal cats, but there was no significant difference between normal and carrier cats.

Hex A and B activities in plasma are shown in Fig. 4. In affected cats, the levels of Hex A and B were markedly low compared with those in normal and carrier cats. However, there was a wide overlap in enzyme activity in plasma Hex A and Hex B between normal and carrier cats. There was no significant difference among the three groups for these two isozymes.

DISCUSSION

The retrospective molecular diagnosis in the present study suggested that the two prior cases (cases 1 and 2) suspected of GM2 gangliosidosis had the 667C>T mutation (Fig. 2). The clinical and pathological features in these two cats were similar to those in 4 affected cats that had previously been diagnosed as having this mutation [8, 9, 23]. The common clinical features summarized from these 6 affected cats are as follows: dwarfism or retarded growth, a clinical course including: onset at about 2 months of age with occurring death at 7 to 10 months of age, and neurological signs such as ataxia, intention tremor and vision defects (Table 2). Clinico-pathological features include: leukocytosis with lymphocytosis and cytoplasmic vacuolation in lymphocyte. These clinical features will allow a tentative practical diagnosis of this disease prior to establishing a definitive diagnosis using enzyme or mutation assays.

These six cats homozygous for the 667C>T mutation were found in widely disparate areas in Japan (Fig. 1). The distance from mark A to each mark (B, C and D) is approximately 25, 10 and 420 km, respectively. Typically, cats move around an area with a maximum radius of 500 m to 1 km. Therefore, it seemed unlikely that cats from the three areas (marks A, B and C) had direct contact with each other. It is also unlikely that these cats were transferred by humans, because Japanese domestic cats are not a recognized pure-breed and are usually out-bred or random-bred in a limited region. The identification of an affected stray cat in mark D suggests that the mutant allele (667C>T) exists widely in the population of Japanese domestic cats in Japan. A common carrier ancestor (founder effect) of these 6 affected cats may have transmitted the allele to multiple offspring a long time ago, and the allele may have been spread across Japan over many generations. The common carrier ancestor might have come to Japan from somewhere in the Eurasian Continent.

In humans, at least 92 specific *HEXA*, 26 *HEXB*, and 4 *GM2A* mutations had been characterized by 2001 [7]. In cats, as indicated above, only 3 *HEXB* and 1 *GM2A* mutations have been identified to date. However, there may be many unidentified mutations causing feline GM2 gangliosidosis in the feline population worldwide. Therefore, it seems to be impossible to definitively diagnose feline GM2 gangliosidosis using only DNA tests based on the mutations identified to date. The enzymic analysis always provides important information for the diagnosis of GM2 gangliosidosis. Thus, enzyme activities in tissues and fluids should be analyzed in each suspected animal, but a DNA test is necessary to detect carriers of feline GM2 gangliosidosis.

Table 2. Major clinical signs of Japanese domestic cats with GM2 gangliosidosis variant 0 (Sandhoff-like disease)*

Age (months)	Major clinical signs
<2	proportional dwarfism or retarded growth, bradykinesia, miosis, appearance of third eyelid
2–3	ataxia (mild to moderate), head and generalized tremor (intension tremor), hypermetria, visual defect
4–5	ataxia (severe), atactic abasia, astasia, blindness
6–7	recumbency, lethargy (mild to moderate), generalized muscular atrophy
7–10	lethargy (severe), lethargic stupor, hypothermia, death

* Clinical signs were summarized from those of 6 affected cats identified in the present and previous studies.

Based on this concept, enzymic analysis was performed in leukocytes and plasma in the present study. As a result, Hex A and Hex B activities in leukocytes and plasma were negligible in affected cats, compared with those in normal and carrier cats (Figs. 3 and 4), leading to the distinct determination of affected cats without molecular diagnosis. For the diagnosis of this feline disease, leukocytes seemed to give a more reliable result than plasma because Hex A and Hex B activities in leukocytes in affected cats differed greatly from those in normal and carrier cats, but the difference was less evident in plasma. For the diagnosis of canine GM2 gangliosidosis as well as the human disease, fluid samples such as serum and cerebrospinal fluid are useful as enzyme sources of Hex activity [24], although fluid samples are not available for the diagnosis of many lysosomal diseases in humans and animals [19], including canine GM1 gangliosidosis [22]. In the present study, it was confirmed that fluid samples such as plasma, which are easily prepared and stored, are also useful for the diagnosis of GM2 gangliosidosis in cats. However, Hex activity was not available for genotyping of carriers with GM2 gangliosidosis, because there was a wide overlap in enzyme activity between normal and carrier cats although the mean value of leukocyte Hex activity in carriers was almost half the normal value. This finding suggests that a DNA test is necessary to detect carriers of feline GM2 gangliosidosis.

Analysis of Hex cannot necessarily be used to make an unequivocal diagnosis of GM2 gangliosidosis, because patients with AB variant and B1 variant that is allelic with Tay-Sachs disease show normal or elevated Hex activities [7]. Actually, in feline AB variant, Hex A activity in tissues was shown to be a little higher than that in control cats [11]. In sporadic canine cases of GM2 gangliosidosis, an increase

in total Hex activity was also reported in a Japanese spaniel [4] and a mixed-breed dog [16], suggesting that these cases may be affected with AB variant. In human and canine B1 variant, Hex A behaves like normal Hex A toward a standard artificial substrate, but is inactive toward the physiological substrate GM2 ganglioside and also toward the sulfated synthetic substrate [7, 17]. In swine GM2 gangliosidosis, an accumulation of GM2 gangliosidosis is associated with elevated Hex activity in serum and reduced Hex activity in tissues [14, 15], suggesting that excessive efflux of Hex from tissues to serum may produce an intracellular enzyme deficiency. Familiarity with which irregular patterns of enzyme deficiencies like these can be present in GM2 gangliosidosis will help increase diagnostic accuracy.

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