Canine GM2-Gangliosidosis Sandhoff Disease Associated with a 3-Base Pair Deletion in the HEXB Gene

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Background: GM2-gangliosidosis is a fatal neurodegenerative lysosomal storage disease (LSD) caused by deficiency of either β-hexosaminidase A (Hex-A) and β-hexosaminidase B (Hex-B) together, or the GM2 activator protein. Clinical signs can be variable and are not pathognomonic for the specific, causal deficiency.

Objective: To characterize the phenotype and genotype of GM2-gangliosidosis disease in an affected dog.

Animals: One affected Shiba Inu and a clinically healthy dog.

Methods: Clinical and neurologic evaluation, brain magnetic resonance imaging (MRI), assays of lysosomal enzyme activities, and sequencing of all coding regions of HEXA, HEXB, and GM2A genes.

Results: A 14-month-old, female Shiba Inu presented with clinical signs resembling GM2-gangliosidosis in humans and GM1-gangliosidosis in the Shiba Inu. Magnetic resonance imaging (MRI) of the dog’s brain indicated neurodegenerative disease, and evaluation of cerebrospinal fluid (CSF) identified storage granules in leukocytes. Lysosomal enzyme assays of plasma and leukocytes showed deficiencies of Hex-A and Hex-B activities in both tissues. Genetic analysis identified a homozygous, 3-base pair deletion in the HEXB gene (c.618-620delCCT).

Conclusions and Clinical Importance: Clinical, biochemical, and molecular features are characterized in a Shiba Inu with GM2-gangliosidosis. The deletion of 3 adjacent base pairs in HEXB predicts the loss of a leucine residue at amino acid position 207 (p.Leu207del) supporting the hypothesis that GM2-gangliosidosis seen in this dog is the Sandhoff type. Because GM1-gangliosidosis also exists in this breed with almost identical clinical signs, genetic testing for both GM1- and GM2-gangliosidosis should be considered to make a definitive diagnosis.

Key words: β-hexosaminidase; Deletion; Dog; Lysosomal storage disease; Neurologic disorder.

Gangliosidoses are neurodegenerative lysosomal storage diseases (LSD) that, as the name indicates, are disorders caused by accumulation of ganglioside lipids in lysosomes. In mammals, they occur as 2 different disorders with various subtypes, with overlapping phenotypes, and have been characterized in several species, including dogs. GM1-gangliosidosis is caused by a deficiency of acid beta-galactosidase (β-GAL; EC 3.2.1.23), which results in progressive and degenerative cerebellar dysfunction. It has been described in Shiba Inu dogs and is characterized by ataxia, intention tremor, dysmetria, progressive inability to stand, corneal clouding, and muscle rigidity. Age of onset is between 5 and 6 months of age, and death generally occurs by 14–15 months of age. A little more than 1% of Shiba Inus in Japan are heterozygous for the disease variant. Disease frequency is unknown in the United States, but GM1-gangliosidosis should be included in the differential diagnosis in a Shiba Inu presenting with progressive cerebellar signs beginning at a juvenile age. The Shiba Inu presented here showed classical signs of GM1-gangliosidosis but normal concentration of beta-galactosidase, suggesting a different cause.

The GM2-gangliosidoses are a group of neurodegenerative lysosomal storage diseases caused by deficiencies in either β-hexosaminidase (HEX, EC 3.2.1.52) type A (Hex-A), β-hexosaminidases A and B (Hex-A and Hex-B) together, or the GM2 activator protein that is necessary for Hex-A activity. Hexosaminidase A consists of an

Abbreviations:
- 4MU: 4-methylumbelliferyl
- 4MU-GlcNAc: 4-methylumbelliferyl-β-D-N-acetyl-glucosaminide
- 4MU-GlcNAcS: 4-methylumbelliferyl-2-acetamido-2-deoxy-6-sulfoglucopyranoside
- bp: base pair
- CSF: cerebrospinal fluid
- GM2AP: GM2-ganglioside activator protein
- HEX: β-hexosaminidase
- kb: kilo base pairs
- Leu: leucine
- LSD: lysosomal storage disease
- MPS: mucopolysaccharidosis
- MRI: magnetic resonance imaging
- OMIM: Online Mendelian Inheritance in Man
- PCR: polymerase chain reaction
- SNP: single nucleotide polymorphism

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alpha subunit coded by the \textit{HEXA} gene and a beta-subunit coded by the \textit{HEXB} gene. Hexosaminidase B, on the other hand, consists of 2 beta subunits. Therefore, defects in \textit{HEXB} will lead to deficiencies in both Hex-A and Hex-B (0 variant), whereas defects in \textit{HEXA} will only lead to a deficiency in Hex-A and a compensatory increase in the Hex-B enzyme (B variant; Fig 1). The GM2 activator protein (GM2A) is necessary for Hex-A activity and is thought to facilitate transport of substrate from the lysosomal membrane for degradation by the enzyme.\(^1\) Although both Hex-A and Hex-B are produced (variant AB), the absence of the GM2A protein leads to a lack of Hex-A activity.

Clinical signs of GM2-gangliosidoses in dogs resemble those in dogs with GM1-gangliosidosis and include ataxia, intention tremors, and falling with a similar age of onset and lifespan.\(^7\)–\(^10\) Mutations in \textit{HEXA}, \textit{HEXB}, and \textit{GM2A} result in 3 subtypes of GM2-gangliosidosis: type I—B variant Tay-Sachs disease (OMIM\(^a\) #272800) caused by \textit{HEXA} mutations that result in Hex-A deficiency; type II—0 variant Sandhoff disease (OMIM \#268800) caused by \textit{HEXB} mutations that result in both Hex-A and Hex-B deficiency; and type III—AB variant GM2AP deficiency (OMIM \#272750) caused by \textit{GM2A} mutations that result in the absence of GM2AP. In human patients with these deficiencies, there is no correlation between the variable clinical signs and the specific mutation in any of the 3 genes. Therefore, the clinical findings alone are insufficient to determine the underlying defect. Thus, enzyme assays and genetic analysis must be performed to determine the type of GM2-gangliosidosis seen in a given patient.

Here, we report naturally occurring GM2-gangliosidosis Sandhoff disease in a Shiba Inu dog with a newly identified homozygous deletion of 3 adjacent base pairs in the \textit{HEXB} gene.

### Materials and Methods

#### Animals

The affected dog, a 14-month-old, female intact Shiba Inu, was obtained from a local pet store at 2 months of age, at which time the dog appeared healthy. Clinical signs began at 9 months of age and included gradually decreasing coordination, difficulty standing and walking, tremors, and ataxia. The dog was referred to the Section of Neurology at the Veterinary School of the University of Pennsylvania for further evaluation. A physical examination, a CBC, CSF analysis, and brain MRI were performed. These studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

#### MR Imaging

Brain MRI was performed with a 1.5-Tesla GE unit under general anesthesia. T2-weighted images, fluid attenuation inversion recovery, gradient echo, diffusion-weighted imaging, and T1-weighted images with and without contrast\(^b\) were obtained.

#### Biochemical Analysis

Blood in EDTA was obtained from the affected dog and from an age-matched clinically healthy, unrelated dog (normal control). Plasma was separated by centrifugation. Leukocytes were isolated by dextran density gradient separation.\(^11\) Genomic DNA was purified\(^c\) and quantified\(^d\) from EDTA blood obtained from both dogs. All specimens were stored at \(-80^\circ\text{C}\) until further study.

Peripheral leukocytes and plasma were analyzed for total \(\beta\)-hexosaminidase (Hex) activity (including both type A and type B) using 4-methylumbelliferyl-\(\beta\)-D-N-acetyl-glucosaminide (4MU-GlcNAc, \(2.5\) mM)\(^12\) and for Hex-A activity only using 4-methylumbelliferyl-2-acetamido-2-deoxy-6-sulfo-\(\beta\)-D-glucopyranoside (4MU-GlcNAcS, \(2.5\) mM).\(^13\) Substrate hydrolysis was measured by fluorometric assay with an excitation wavelength of 365 nm and an emission wavelength 450 nm with a Bio-Rad VersaFluor\(^g\) fluorometer.\(^f\) Enzyme specific activity was reported as nM of 4-methylumbelliferyl (4MU) released per milligram of leukocytes protein or per milliliter of plasma, per hour. Because defects in other lysosomal enzyme activities can result in neurologic signs, activities of additional lysosomal enzyme activities were assayed by standard protocols,\(^i\) including \(\beta\)-galactosidase (\(\beta\)-GAL; EC 3.2.1.23 for GM1-gangliosidosis), \(\alpha\)-L-fucosidase (\(\alpha\)-FUC; EC 3.2.1.51 for \(\alpha\)-fucosidosis), \(\beta\)-glucocerebrosidase (\(\beta\)-GCB; EC 3.2.1.45 for Gaucher’s disease), and N-acetyl-\(\alpha\)-D-glucosaminidase (NAGLIU; EC 3.2.1.50 for MPS IIIB). Protein concentrations were determined with the Bio-Rad protein assay kit\(^h\) and expressed as mg/mL.

#### Molecular Analysis

The DNA sequences of 3 genes, \textit{HEXA} on canine chromosome 30, \textit{HEXB} on chromosome 2, and \textit{GM2A} on chromosome 4...
(including protein-coding portions of exons and the exon-intron junction areas) were determined by established protocols involving exon amplification by polymerase chain reaction (PCR) followed by Sanger sequencing at a core facility. A negative PCR control comprised of deionized water instead of genomic DNA was used to monitor for possible contamination. The sequencing results were compared to the published canine genome sequences, NCBI GenBank® RefSeq predicted mRNAs, and Ensembl Can-Fam 3.1 assembly® predicted transcripts (HEXA, RefSeq XM_014109579.1, Ensembl ENSCAF00000028088.3; HEXB, RefSeq XM_005617912.2, ENSECAFT00000035273.2; and GM2A, RefSeq XM_003639011.3, ENSECAFT00000028421.3), as well as to the simultaneously sequenced genomic DNA of a clinically healthy dog. For the HEXB gene, predicted cDNA sequences and the reference canine genome sequence contain only 13 exons. Other mammals have an additional exon at the 5’ end (e.g., humans, mice, cats, and cattle), which appears to be missing in the canine genome sequence. For the HEXB gene and HEXB protein (β-subunit), nucleotide and amino acid positions, respectively, are given with respect to the canine Ensembl entry ENSECAFT00000035273.2 transcript and its predicted protein.

The predicted HEXB amino acid surrounding the DNA deletion site of the affected dog was aligned and compared to the locus of a normal dog and other vertebrates using the default parameters of the Jotun Heim algorithm in the Megalign program of the DNASTRAX® Sequence Analysis Package. Sequences used in this alignment were ENSECAFT00000035273.2 (dog), NP_001009333 (cat), NP_000512 (human), NP_001306373 (macaque), NP_001069978 (cow), NP_999086 (pig), NP_0034552 (mouse), NP_00111946 (rat), NP_001135106 (Atlantic salmon), and NP_001279725 (elephant shark).

The effect of the single amino acid deletion on protein function was assessed by protein variation effect analyzer software (PROVEAN), which can assess both missense and deletion variants. The PROVEAN deleterious prediction score cutoff point is –2.5 (≥–2.5: neutral; <–2.5: deleterious).

Results

Clinical Examination

On physical examination, the affected dog had an ideal body condition score of 9 and was bright, alert, and responsive. Neurologic evaluation disclosed severe cerebellar ataxia, bilateral lack of menace response, severe intention tremors, and inability to stay standing for any period of time. The CBC disclosed a low hemoglobin concentration at 11.4 g/dL (normal, 14.1–20.1 g/dL) and purple staining granules in the cytoplasm of the lymphocytes. The CSF analysis indicated a normal cell count, but many prominent purple granules in the cytoplasm of Wright-Giemsa-stained leukocytes were observed (Fig 2).

Brain MRI Scan

The MRI analysis of the affected dog’s brain identified prominent cerebellar folia (Fig 3A). Dilated olfactory ventricles were present (Fig 3B), and a loss of white and gray matter distinction was noted in the cerebrum (Fig 3C) with white matter isointense to gray matter. Dilated sulci of the occipital and temporal lobes also were present (Fig 3D). These changes indicated cerebral atrophy. The white matter of the cerebellum was hyperintense to the gray matter (Fig 3E). These white matter changes and atrophy were consistent with demyelination, edema, or inflammation, which can be seen with metabolic diseases, toxic disorders, or viral inflammation.

Lysosomal Enzyme Assays

The activities of β-GAL, total Hex, and Hex-A in plasma and leukocytes were determined by a fluorescence assay using 4MU-containing substrates. The activities of 3 other lysosomal enzyme activities also were determined because their deficiencies can result in similar neurologic signs (α-FUC for α-fucosidosis, β-GBA for Gaucher’s disease, and NAGLU for MPS IIIB).

Enzyme assay results demonstrated abnormally low total Hex activity in both plasma and white blood cells from the affected dog (2.3% and 2.4% of normal control, respectively). To further confirm the decreased activity of Hex-A, the samples were retested with a second, more specific, sulfated substrate, 4MU-GlcNAcS. The Hex-A activities in both plasma and white blood cells were <5% of that of the normal controls. All other lysosomal enzyme activities were within reference ranges (Table 1).

Variant Discovery

The canine HEXB gene is located on canine chromosome 2 with 13 exons encoding the Hex β-subunit protein, which consists of 454 amino acid residues. Comparison of the HEXB exon sequences from the affected dog’s genomic DNA to the published canine genome sequence, as well as to the simultaneously sequenced HEXB in an unrelated, clinically healthy dog, identified a homozygous sequential 3-bp deletion in exon 7 (c.618_620delCCT, corresponding to CanFam3.1 genome position CFA2: 57,243,656–8), which would result in the removal of 1 amino acid, a leucine.

Fig 2. Representative mononuclear cell from CSF of the affected dog. Prominent purple granules are present in the cytoplasm. (Wright-Giemsa stain, bar = 10 μm).
at position 207 (p.Leu207del, Fig 4, Table 2). A previously identified single nucleotide polymorphism (SNP) was identified in exon 8 of both the normal and affected dog (c.G825A, SNP25980623), resulting in a silent variation (p.Lys275Lys) when compared to the published canine genomic sequence (Table 2).

The HEXA gene (14 exons encoding the Hex α-subunit polypeptide chain of 529 amino acid residues) and
GM2A gene (4 exons encoding the GM2A protein of 196 amino acid residues) were sequenced, and no variants were discovered in the coding regions of HEXA. However, 2 previously identified SNPs, both in exon 4 of GM2A (c.T755A/SNP542501 is a aspartic acid-to-glutamic acid missense substitution at position 154 [D154E] where humans and other primates have a glutamic acid residue; c.A856G/SNP542502 is a lysine-to-arginine missense substitution at position 188 [K188R], the ninth amino acid from the carboxyl terminus of the protein), were found in genomic DNA from both the normal and affected dog when compared to the published canine sequences (Table 2).

Protein Structure Prediction and Conservation

Alignments of the amino acid sequence of the Hex β-subunit peptide from multiple species including humans indicated that the deleted leucine at position 207 is in a highly conserved region (Fig 5). The PROVEAN score of −13.66 predicts that the deletion of leucine at p207 is deleterious to the of the Hex beta-subunit protein. The PROVEAN scores for the previously identified variants in the GMA2 gene are −0.339 for D154E and −2.27 for K188R, predicting that these 2 missense polymorphisms do not affect protein function.

Discussion

GM1-gangliosidosis, another neurologic genetic LSD with similar clinical signs to those seen in this affected dog, has been reported previously in Shiba Inu dogs. Additionally, several LSDs, such as α-fucosidosis, Gaucher’s disease, and MPS IIIB, clinically are nearly indistinguishable. To discriminate among the various possible LSDs, specific lysosomal hydrolase assays for each disease were performed (as is routinely done in this laboratory to quickly screen for a deficiency). The normal activity of acid β-GAL ruled out GM1-gangliosidosis in the affected dog. Normal activities of α-FUC, β-GBA, and NAGLU (Table 2) likewise ruled out the possibilities of α-fucosidosis, Gaucher’s disease, and MPS IIIB, respectively. In contrast, the remarkably decreased activities of total Hex led to a diagnosis of GM2-gangliosidosis, with deficiency of both Hex-A and Hex-B activities, implicating a DNA variation in the HEXB gene as the most likely molecular defect.

A few cases of GM2-gangliosidosis have been described in dogs. The earliest documentation of ganglioside storage was made in German shorthaired pointers, but the type was never determined and the underlying defect unknown. At the time the study was published, only male dogs were known to be affected, but the number of affected dogs was small (n = 6; with a 1.56% chance of this observation happening randomly). The clinical signs seen in these dogs were similar to those of the Shiba Inu in our study. Until 6 months of age, the dogs trained normally after which their cognitive functions appeared to decline. By 1 year of age, ataxia set in and the dogs appeared to be blind. Because of the degenerative nature of the disease, humane euthanasia was performed by 18 months of age.

More recently, a Japanese Chin (Japanese Spaniel) was described with a slightly later onset of similar clinical signs, but histologic and ultrastructural studies confirmed GM2-gangliosidosis. Additional studies were suggestive of GM2A deficiency or type AB disease.

![Image](4)

Fig 4. The electrophoreogram of a partial region of exon 7 of canine HEXB from (A) a clinically healthy dog and (B) the dog affected with GM2-gangliosidosis Sandhoff disease. Comparison of the HEXB sequence from the DNA from the clinically healthy dog with that of the affected dog revealed a homozygous, 3-adjacent-base pair deletion (c.618-620delCCT). This deletion removed 1 amino acid (p.Leu207-del) from the β-subunit peptide chain of Hex β.
because Hex-A and Hex-B activities were thought to be increased. However, a single base pair substitution in HEXA predicting an amino acid substitution was discovered implicating the Tay-Sachs variant of GM2-gangliosidosis.

In 2002, GM2-gangliosidosis was described in a male Golden Retriever with the same clinical signs as the Shiba Inu described in our study and death occurred by 15 months of age. Activities of both Hex-A and Hex-B were decreased, suggesting Sandhoff type of GM2-gangliosidosis, but the molecular defect was not elucidated.

Several years later, Toy Poodles (2 females and 1 male) with a common ancestor were described with clinical signs of falling over, head tremors, ataxia, and apparent blindness beginning around 9 months of age. The progressive nature of the disease leads to death between 18 and 23 months of age in all affected dogs. Blood smears disclosed vacuolated lymphocyte cytoplasm, and both Hex-A and Hex-B activities were <3% of normal, which was suggestive of GM2-gangliosidosis. A single base pair deletion in exon 3 of HEXB was discovered, resulting in a frameshift and a premature stop codon and confirming Sandhoff type of GM2-gangliosidosis. The overall frequency of heterozygotes was found to be only 0.2% in the Japanese Toy Poodles, and visual examination of the pedigree of affected dogs suggested that the disease was limited to 1 particular line of Toy Poodles.

Lastly, a mixed-breed dog was described with clinical signs and age of onset reminiscent of GM2-gangliosidosis. Both blood Hex-A and Hex-B activities were well below normal, suggesting the Sandhoff type. However, DNA from this dog tested negative for the Toy Poodle variant.

The affected dog in our study was born as a healthy puppy and began showing signs of neurologic problems at 9 months of age. The presentation of Sandhoff disease in this dog is similar to the other 3 forms of the disease in dogs. The onset of clinical signs was between 9 and 12 months. The clinical signs included stiff gait, loss of balance, difficulty walking, frequent falling, ataxia, and tremors. All reported Sandhoff disease-affected dogs, including our affected dog, had abnormal cytoplasmic vacuoles or granules in white blood cells and in cells from the CSF. Major brain MRI lesions were consistent with cerebral and cerebellar atrophy, in particular the widened cerebral sulci and dilated olfactory ventricles. Together with the hyperintensity of the cerebellar white matter, and diffuse blurring of white and gray matter distinction, the changes indicate similar myelin deterioration as observed in Sandhoff disease in affected humans and other dogs.

The β-subunits of the HEX protein in humans have 2 domains: domain I from amino acid residues 50 to 201, and domain II from residues 202 to 556, which serve as

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Table 2. Summary of variations in the coding regions of canine HEXA, HEXB, and GM2A genes.

| Gene | Chromosome (total exons) | Product | Variation on Exon | Nucleotide | Codon | Amino Acid |
|------|--------------------------|---------|-------------------|------------|-------|------------|
| HEXA | 30 (14)                  | α subunit | No mutations    |            |       |            |
| HEXB | 2 (13)                   | β-subunit | c.615-617delCCT   | CCT > del  | p.207delLeu |
| GM2A | 4 (4)                    | GM2AP    | c.G825A          | AA>G       | Lys275Lys |
|      |                          |          | c.T755A          | GA>G       | Asp154Glu |
|      |                          |          | c.A856G          | AA>G       | Lys188Arg |

Variation found only in the proband (nucleotide and amino acid changes in bold).

Varations found in both the proband and a clinically healthy dog.

Silent mutations, which resulted in no change in translation.

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Fig 5. Multispecies alignment of HEXB protein sequences near the L206del variant. The top line shows the predicted canine sequence. Other lines have dots when the sequence is identical to dog, with the actual amino acid shown when it differs from the dog sequence. The dash in the affected (AF) dog sequence represents the deleted leucine residue. The C underlined in the top row denotes the location of the closest disease-causing variant in humans. Numbers on the right are the amino acid position of the last residue of each sequence.
the enzyme catalytic site. The dog amino acid Leu 207 (orthologous to human residue Leu 306) is predicted to reside in domain II of the HEXB protein, between beta sheet 3 and alpha helix 3, within a highly evolutionarily conserved region (Fig 5). In humans, the closest disease-associated change is a nearby missense mutation (C309Y) that was identified in a patient with an adult onset form of Sandhoff disease.

GM2-gangliosidosis in dogs is inherited as an autosomal recessive trait. If the disease-associated variant identified here is or becomes more common in the breed, identification of clinically normal carriers would be important for controlling the incidence of GM2-gangliosidosis in this dog breed. Since the initial case, we have seen a similarly affected Shiba Inu, also homozygous for this 3-bp deletion. Therefore, DNA testing for both GM1- and GM2-gangliosidosis should be used to make a definitive diagnosis in suspicious cases and to guide breeding decisions. In addition, another report recently was published describing 2 Shiba Inus with GM2-gangliosidosis with the same mutation, suggesting that there are more heterozygote animals in the population than previously thought.

Currently, there is no known cure for GM2-gangliosidosis, and the affected dogs gradually deteriorate and die (or are euthanized for humane reasons) at a young age. This naturally occurring dog model of GM2-gangliosidosis could be used to develop a better understanding of clinical and biochemical consequences, molecular pathogenesis, and for developing new therapeutic strategies for neurodegenerative disorders in humans.

Footnotes

a Online Mendelian Inheritance in Man, https://www.omim.org/
b Magnevi.st, Bayer
c QIAtamp DNA Blood Mini Kit, QIAGEN, Valencia, CA
d NanoDrop 2000, UV-Vis Spectrophotometer, Thermo Scientific, Wilmington, DE
e Cat# M2133, Sigma-Aldrich, St. Louis, MO
f Cat# M0662, Sigma-Aldrich, St. Louis, MO
b Bio-Rad Laboratories, Hercules, CA
c http://www.ncbi.nlm.nih.org
f http://www.ensembl.org/Caniis_familiaris
h Applied Biosystems, Life Technologies, Grand Island, NY
b DNASTAR, Inc., Madison, WI
j The J. Craig Venter Institute, Rockville, MD, http://sift.jcvi.org

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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.

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