GM2 Gangliosidosis in Shiba Inu Dogs with an In-Frame Deletion in HEXB

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Consistent with a tentative diagnosis of neuronal ceroid lipofuscinosis (NCL), autofluorescent cytoplasmic storage bodies were found in neurons from the brains of 2 related Shiba Inu dogs with a young-adult onset, progressive neurodegenerative disease. Unexpectedly, no potentially causal NCL-related variants were identified in a whole-genome sequence generated with DNA from 1 of the affected dogs. Instead, the whole-genome sequence contained a homozygous 3 base pair (bp) deletion in a coding region of HEXB. The other affected dog also was homozygous for this 3-bp deletion. Mutations in the human HEXB ortholog cause Sandhoff disease, a type of GM2 gangliosidosis. Thin-layer chromatography confirmed that GM2 ganglioside had accumulated in an affected Shiba Inu brain. Enzymatic analysis confirmed that the GM2 gangliosidosis resulted from a deficiency in the HEXB encoded protein and not from a deficiency in products from HEXA or GM2A, which are known alternative causes of GM2 gangliosidosis. We conclude that the homozygous 3-bp deletion in HEXB is the likely cause of the Shiba Inu neurodegenerative disease and that whole-genome sequencing can lead to the early identification of potentially disease-causing DNA variants thereby refocusing subsequent diagnostic analyses toward confirming or refuting candidate variant causality.

**Key words:** Autofluorescent storage bodies; Lysosomal storage disease; Neuronal ceroid lipofuscinosis; Sandhoff disease; Whole-genome sequence.

**Abbreviations:**

| Abbreviation | Description |
|--------------|-------------|
| NCL | Neuronal ceroid lipofuscinosis |
| PCR-RFLP | PCR-restriction fragment length polymorphism |

likely responsible for this disease and to reach a highly probable, but unexpected, diagnosis.

**Methods**

The DNA from 1 affected Shiba Inu (Dog A) was submitted to a commercial sequencing center for PCR-free library preparation and sequencing with a commercial sequencer that produced paired-end 150-bp reads. Details about the pipeline used to process and analyze this data set are available in the supplementary material. The PCR primers 5'-AAATAGTATAATCATGTG-3’ and 5'-CCCAATACATTACTCTGCTA-3’ were used to produce amplicons containing a candidate variant identified in the whole-genome sequence. These amplicons were analyzed by Sanger sequencing for validation and by PCR-restriction fragment length polymorphism (PCR-RFLP) with MnlI for genotyping archived Shiba Inu DNA. Previously described procedures were used for fluorescence and electron microscopy and for thin-layer chromatography and enzymology.

**Clinical Findings**

Dog A, a spayed female Shiba Inu, exhibited progressive neurologic signs starting with generalized tremors and altered tail carriage (tail down) when approximately 12 months old. As the disease progressed, the severity of the tremors increased and the dog exhibited marked cerebellar ataxia, spastic tetraparesis, anxiety, decreased appetite, decreased responsiveness to verbal commands,
Dog B also was a spayed female Shiba Inu. Similar to Dog A, the first clinical signs in Dog B appeared at about 12 months of age and included situational anxiety, inability to climb up or down stairs, a wide-based stance, severe generalized tremors, and the same alteration in tail carriage shown by Dog A. During a neurologic examination at 15 months of age, Dog B had normal proprioception but showed intention tremors and the same alteration in tail carriage relative to gray matter. Because of the progression of neurologic signs, the dog was euthanized at approximately 17 months of age.

Molecular-Genetic Diagnosis

The clinical signs and fluorescent microscopic results were consistent with a diagnosis of NCL. Because whole-genome sequencing previously had proven to be an effective strategy for identifying genetic variants responsible for NCL, a whole-genome sequence was generated with DNA from Dog B. The resulting sequence data had 31-fold average coverage of the CanFam3.1 reference assembly and are available in the National Center for Biotechnology Information Sequence Read Archives (accession SRS1692083). As we have done previously, the aligned reads from the whole-genome sequence were scanned for rare variants in or near the known exons of the 13 genes currently associated with human NCL: PPT1, TPP1, CLN3, CLN5, CLN6, MFSD8, CLN8, CTSD, DNAJC5, CTSE, ATP13A2, GRN, and KCTD7. Unexpectedly, we did not find plausible candidate variants. In addition, the alignment was checked for a GLBI frameshift mutation, chr23:3,796,317delC (CanFam 3.1), known to cause a GM1 gangliosidosis in the Shiba Inu breed (OMIA 000402-9615).3 Because the clinical signs suggested neuronal ceroid lipofuscinosis (NCL) as a possible cause, blood and other tissue samples were shipped to the University of Missouri for further analysis. Fluorescence microscopy of substantia nigra sections from both affected dogs identified massive accumulations of yellow-emitting autofluorescent storage bodies in neurons of the cerebellum (Fig 2A), the cerebral cortex (Fig 2B), and the retina (Fig 2C).

Fig 1. Pedigree shows consanguineous familial relationships for the two affected Shiba Inu, Dog A and Dog B. The dog marked with an asterisk contributed genetic material to both parents of both affected dogs. Bold lines show the shortest possible pathway for the inheritance of the causal allele from an ancestor to the homozygous affected dogs. Nonetheless, we do not have DNA from the ancestors to verify this pathway, and additional consanguineous relationships among earlier ancestors are likely. Thus, Dog A and Dog B may have obtained their homozygous mutant allele by alternative inheritance pathways.
HEXB deletion among the Shiba Inu, all 40 Shiba Inu represented in the University of Missouri DNA repository were genotyped by PCR-RFLP for this deletion. Thirty-seven of the tested Shiba Inu were homozygous for the reference allele; the other 3 were heterozygotes. These heterozygotes were born in 2002, 2004, and 2007. There are no known familial relationships among them or between them and the 2 affected Shiba Inu described here. This observation suggests that the 3-bp HEXB deletion may be rare but widely distributed in the Shiba Inu breed.

Biochemical and Electron Microscopic Confirmation

The HEXB deletion predicts the loss of a leucine residue (p.Leu317del; XP_005617969.1) in the encoded β-subunit of the β-hexosaminidase enzyme complex. A leucine residue occurs at a homologous position in β-subunits from 53 other mammalian species (see Figure S1). A functional β-subunit provides essential glycosidic activity to the β-hexosaminidase enzyme complex that removes the terminal N-acetyl-galactosamine from GM2 ganglioside, an essential step in the lysosomal degradation of gangliosides. Genetic variants in human HEXB can result in a β-hexosaminidase deficiency called Sandhoff disease and cause the accumulation of GM2 ganglioside.11

Thin-layer chromatography was used to determine whether GM2 ganglioside had accumulated in brain gray matter from 1 of the affected Shiba Inu (Dog B). The resulting thin-layer chromatogram is shown in Fig. 3. An extract from the brain of a human patient with GM2 gangliosidosis known to contain GM2 ganglioside as the major component was fractionated in lane 1. The brain extract in lane 3 was from a human GM1 gangliosidosis patient. GM1 ganglioside was the major component in this extract. Lane 4 contained a normal bovine brain extract and served to show that GM1 and GM2 gangliosides do not accumulate in healthy brains. The extract in lane 2 was from one of

Fig 2. Fluorescence micrographs of unstained sections of cerebellum (A), cerebral cortex (B), and retina (C) from an affected Shiba Inu. In the cerebellum, autofluorescent intracellular inclusions were present in both the Purkinje cell (p) and molecular (m) layers. Neurons throughout the cerebral cortex gray matter had abundant autofluorescent inclusions. In the retina, the most pronounced accumulation of autofluorescent material was seen in the ganglion cell layer (g), but some accumulation was also present in the inner nuclear layer (inl). Bar in (A) indicates the magnification for micrographs (A) and (B).

Fig 3. Thin-layer chromatographic fractionation of brain gangliosides extracted from the gray matter of a human patient who died from Tay–Sachs disease (lane 1), the gray matter of Dog B, the affected Shiba Inu dog (lane 2), the gray matter of a human patient who died with GM1 gangliosidosis (lane 3) and gangliosides extracted from a normal bovine brain obtained from a commercial source (lane 4). The most abundant component in lane 1 is GM2 ganglioside (GM2). In lane 2, the most abundant component co-migrated with the GM2 ganglioside in lane 1 and not with GM1 ganglioside (GM1), the most abundant component in lane 3. Only trace amounts of GM2 ganglioside were detected in Lane 3, and none was detected in extracts from the normal brain in lane 4.
the affected Shiba Inu (Dog B). The migration of the major component in this lane matched that in lane 1, indicating that it is also GM2 ganglioside and that the dog had a GM2 gangliosidosis.

Besides HEXB, GM2 gangliosidosis can be caused by genetic variants in 2 other genes: HEXA that encodes the α-subunit of the β-hexosaminidase enzyme complex and GM2A that encodes GM2 activator protein. Although the activator protein does not possess intrinsic enzymatic activity, its presence is required for efficient enzymatic degradation of GM2 ganglioside by β-hexosaminidase.12 The α- and β-subunits of β-hexosaminidase are enzymatically active as homo- or heterodimers. The 3 genetic causes of GM2 gangliosidosis can be enzymologically distinguished with synthetic substrates that identify the source of the residual hexosaminidase activities in patients’ tissues.13 Patients with GM2 gangliosidosis caused by a GM2A deficiency have residual activity from both the α- and β-subunits. Thus, they are classified as AB variants. Human patients with HEXA deficiency (known as Tay–Sachs disease) are considered B variants because they have only β-subunit activity. Homodimers of the α-subunit are unstable and heat labile. Patients with Sandhoff disease (HEXB deficiency) who lack the β-subunit necessary to form stable heterodimers can only form unstable α-subunit homodimers and thus have subnormal activities from both the α- and β-subunits.11 These patients are considered to be 0 (zero) variants.

Table 1 contains a summary of the genetic causes, the subunit activities, and the associated nomenclature for the 3 causes of GM2 gangliosidosis.

We used previously described procedures2 and the synthetic substrate, 4-methylumbelliferyl n-acetyl-β-d-glucosaminide, to estimate total β-hexosaminidase activity in cerebral cortical gray and white matter extracts. We also measured β-galactosidase activity in these extracts as a control. As indicated in Table 2, the total β-hexosaminidase activities in the extracts from Dog B were markedly decreased compared to 2 control dogs. Furthermore, the β-hexosaminidase activities in the extracts from Dog B were completely heat labile as would be expected for α-subunit homodimers. These results are similar to those observed with 0 variant or Sandhoff disease patients and provide further evidence that the HEXB deletion identified by whole-genome sequencing is responsible for the progressive neurodegeneration in the 2 affected Shiba Inu dogs.

Table 1. Types of GM2 Gangliosidosis

| Disease                  | Gene   | Encoded Protein               | Residual Activity | Variant |
|--------------------------|--------|--------------------------------|-------------------|---------|
| Tay–Sachs Disease        | HEXA   | α-Subunit of β-Hexosaminidase  | β-Subunit         | B Variant |
| Sandhoff disease         | HEXB   | β-Subunit of β-Hexosaminidase  | Minimal           | 0 Variant |
| GM2 Activator Deficiency | GM2A   | GM2 Activator                  | α- and β-Subunits | AB variant |

*Harboring causal variants.

*Based on residual enzymatic activity.

*Minimal because α-subunit dimers are unstable.

Table 2. Enzyme Activities

| Enzyme                  | Enzymatic Activities (nmol/h/mg protein) |
|-------------------------|-----------------------------------------|
|                         | Affected Dog B | Control Dog 1 | Control Dog 2 |
|                         | Gray Matter | White Matter | Gray Matter | White Matter | Gray Matter | White Matter |
| β-galactosidase*        | 269        | 174         | 67.1       | 25.5        | 58.9        | 31.8        |
| β-hexosaminidase (%)    | 15.1 (100%) | 11.3 (100%) | 663 (77%)  | 363 (72%)   | 669 (79%)   | 427 (79%)   |

*Run as control.
required for inclusion among the diverse diseases classified as NCLs. Nonetheless, in a few cases, disorders with these characteristics have been classified as other types of lysosomal storage diseases because of the biochemical composition of the associated storage granules or the nature of the associated enzyme deficiency or both. Among these are GM1 gangliosidosis and mucopolysaccharidosis IIIA in humans and α-mannosidosis in mice. In addition, there is an example in dogs. When American Staffordshire Terriers and Pit Bull Terriers with a recessively inherited, adult-onset, progressive neurodegenerative disease were found to

Fig 4. Electron micrographs of storage material in the cerebral cortex (A and B), cerebellar molecular layer cell (C), and retinal ganglion cells (D) of Dog B. Representative storage bodies are indicated by arrows. In cerebral cortical neurons, the storage material consisted of both concentric whorls of membrane-like structures (A) and membrane-bound vacuole-like inclusion bodies (B). In the cerebellum (C), the storage bodies contained more irregularly arranged membrane-like material. The ultrastructural appearance of the cerebellar storage bodies was similar in both the molecular layer and the Purkinje cells. In the retinal ganglion cells (D), the storage material consisted primarily of concentric whorls of membrane-like structures.

Fig 5. Fluorescence micrographs of unstained cryostat sections of cerebellum (A) and cerebral cortex (B) from a Japanese Chin dog with GM2 gangliosidosis due to a homozygous HEXA nonsynonymous substitution. In the cerebellum, autofluorescent inclusions were present primarily in the Purkinje cells (arrows in A) in the Purkinje cell layer (p), although weak fluorescent cells were also present in the molecular layer (m). No autofluorescence was observed in the granular cell layer (g). Neurons throughout the cerebral cortex gray matter of the parietal lobe had abundant autofluorescent inclusions (arrows in B). Bar in (A) indicates the magnification for micrographs (A) and (B).
accumulate autofluorescent storage bodies in hippocampal and cerebellar neurons, their disease was classified as an NCL. This classification remained unchanged with the identification of the causal genetic variant in ARSG, a gene with a biologic function that had not yet been determined. Subsequently, it was shown using Arg-knockout mice that the natural substrate for the encoded enzyme was heparan sulfate, a mucopolysaccharide, and that the storage bodies were rich in partially degraded heparan sulfate. Thus, the disease caused by ARSG deficiency is now considered to be a mucopolysaccharidosis. Similarly, the Shiba Inu of our case report were thought to have NCL until we determined that brain extracts from 1 of the dogs contained accumulated GM2 ganglioside and had a β-hexosaminidase deficiency, thus classifying the disorder as a gangliosidosis.

Ours may be the first report that storage bodies associated with GM2 gangliosidosis are autofluorescent. In fact, at least 1 report states that storage bodies from human patients with GM2 gangliosidosis do not autofluoresce. Thus, we examined tissue from a previously reported case of GM2 gangliosidosis in a Japanese Chin dog caused by a HEXA mutation (OMIA 001461-9615). As with the results reported here, the storage bodies in the tissue from the dog with the HEXA mutation were autofluorescent (Fig. 5).

Comparisons with Other Gangliosidoses in Dogs

Although a GM1 gangliosidosis has been recognized in the Shiba Inu for >15 years, ours is the first report of a GM2 gangliosidosis in this breed. GM2 gangliosidoses have been reported in a mixed breed dog and in members of other dog breeds including German Shorthaired Pointer, Japanese Chin, Golden Retriever, and Toy Poodle. The Toy Poodle, Golden Retriever, and the mixed breed dog were classified as 0 variant (OMIA 001462-9615). The 1-bp HEXB frameshift variant responsible for the 0-variant gangliosidosis in Toy Poodles is different from the 3-bp in-frame deletion that we found in the affected Shiba Inu dogs. The molecular-genetic causes for the 2 other 0-variant gangliosidoses have not been reported.

Conclusions

Although not yet routine, the sequencing and analysis of whole genomes generated from patient DNA is becoming an increasingly important strategy for diagnosing inherited diseases in humans. Technical improvements in massively parallel DNA sequencing have resulted in the development of a sequencing instrument capable of generating deep sequence coverages of mammalian genomes for <$2,000. Although initially licensed exclusively for the sequencing of human genomes, this instrument can now be used to sequence domestic animal genomes. With access to this technology, it became economically feasible for us to make use of whole-genome sequencing early in our efforts to identify the molecular-genetic cause of the neurodegeneration in these Shiba Inu. Analysis of the whole-genome sequence from 1 of the affected dogs identified a potentially causal candidate variant. This finding enabled us to proceed with more traditional diagnostic techniques (e.g., thin-layer chromatography, enzymology, electron microscopy) that were chosen specifically to confirm or refute the causality of the candidate variant. Thus, in this case study, whole-genome sequencing was an efficient and cost-effective procedure that led to the identification of the Shiba Inu disease as a GM2 gangliosidosis caused by a 3-bp deletion in HEXB. Once impediments such as long turn-around times, low causal variant discovery rates, and inadequate data storage facilities have been overcome, we expect whole-genome sequencing to become an increasingly important tool for diagnosing heritable animal diseases.

Footnotes

* McDonnell Genome Institute, Washington University, St Louis, MO 63108
* HiSeq X Ten Sequencing System, Illumina, Inc., San Diego, CA 92122
* Paw Print Genetics, 850 E. Spokane Falls Blvd. Suite 200, Spokane, WA 99202
* VetGen, 3728 Plaza Drive, Suite 1, Ann Arbor, MI 48108
* Sigma-Aldrich, 3300 S 2nd St, St. Louis, MO 63118

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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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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Alignments of β-hexosaminidase β-subunit amino acids sequences from 54 mammalian species predicted from codons surrounding the deleted Leu137 codon in the affected Shiba Inu.

Table S1. Rare variants predicted to change the primary sequence of the gene product.