Published in final edited form as:

Mol Genet Metab. 2010 August ; 100(4): 349–356. doi:10.1016/j.ymgme.2010.04.009.

A mutation in canine *PPT1* causes early onset neuronal ceroid lipofuscinosis in a Dachshund

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Abstract

The neuronal ceroid lipofuscinoses (NCLs) are lysosomal storage diseases characterized by progressive neurodegeneration and accumulation of autofluorescent storage granules. A 9 month old Miniature Dachshund presented with NCL-like signs that included disorientation, ataxia, weakness, visual impairment and behavioral changes. Neurons throughout the CNS contained autofluorescent lysosomal inclusions with granular osmiophilic deposit (GROD) ultrastructure characteristic of classical infantile NCL (INCL). Human INCL is an autosomal recessive disorder that results from mutations in *PPT1*, a gene that encodes the enzyme palmitoyl protein thioesterase 1 (PPT1; EC 3.1.22). Resequencing of *PPT1* from the affected dog revealed that the dog was homozygous for a single nucleotide insertion in exon 8 (*PPT1* c.736_737insC), upstream from the His289 active site. Brain tissue from this dog lacked PPT1 activity. The sire and dam of the propositus were heterozygous for the c.736_737insC mutation; whereas, 127 unrelated Dachshunds were homozygous for the wild-type allele. This is the first reported instance of canine NCL caused by a mutation in *PPT1*.

Keywords

INCL; Palmitoyl protein thioesterase; Canine model; Neurodegeneration

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1. Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a group of heritable, lysosomal storage disorders characterized by progressive neurodegeneration accompanied by the accumulation of autofluorescent lysosomal storage granules in the central nervous system and other tissues [1]. The NCLs are classified clinically by age of onset into infantile (INCL), late-infantile, juvenile and adult onset forms. They are additionally categorized according to the defective genes associated with the disease. To date eight genes (*PPT1*, *TPP1*, *CLN3*, *CLN5*, *CLN6*, *CLN8*, *CTSD*, and *MFSD8*) have been definitively associated with NCL in human patients [2–9]. All forms of NCL result in progressive neurodegeneration of the central nervous system. Affected individuals suffer vision loss, progressive motor and cognitive decline, and seizures. The disease usually culminates in a persistent vegetative state and premature death [1]. There is currently no effective treatment for any of the NCLs.

The most common form of INCL is caused by mutations in the *PPT1* gene, located on human chromosome 1p32, which encodes the enzyme palmitoyl protein thioesterase 1 (PPT1) [2]. PPT1 catalyses the removal of the fatty acid palmitate from its thioester linkage to serine residues in S-acylated proteins [10,11]. Palmitoylation imparts a local hydrophobic character to modified proteins, promoting interactions with membranes or other hydrophobic proteins which are crucial for processes such as membrane anchorage, vesicular transport, signal transduction and the maintenance of cellular architecture [12,13]. PPT1 localizes to lysosomes in peripheral cells, while in cortical neurons it is axonally targeted and is present in synaptosomes and synaptic vesicles [14–17]. Numerous *PPT1* mutations have been reported in patients with NCL [18]. Mutations which cause complete loss of PPT1 activity always cause severe, infantile-onset NCL [19].

Numerous mutations responsible for spontaneous NCL have been documented in domestic and research animals including sheep, cattle, mice and dogs [7,18,20–24]. Mutations in canine orthologs of human *TPP1*, *CLN5*, *CLN8* and *CTSD* have been discovered [25–28]. Two mouse gene knockout models of human INCL, bearing mutations in the mouse ortholog of human *PPT1*, have been created and characterized extensively [29–31]. To date, no instances of canine NCL caused by a mutation in *PPT1* have been reported. Here, we describe the clinical, ultrastructural, molecular and genetic features of a novel form of INCL in a Miniature Dachshund with a mutation in *PPT1*.

2. Methods

2.1 Clinical examination and sample collection

A pet male Miniature Dachshund was the product of an accidental mother/son breeding. After exhibiting progressive neurologic signs for a number of months, the dog was examined at Veterinary Specialists of South Florida at 9 months of age. Regular neurology and ophthalmology examinations were subsequently performed over a period of 5 months. On the basis of the clinical signs and the progressive nature of the disease, the dog was suspected of suffering from NCL, and the case was referred to the Comparative Neurology Program at the University of Missouri. The owners completed a symptom questionnaire (http://www.caninegeneticdiseases.net/CL_site/SampSurv.pdf) that has been used successfully to identify NCL in a number of canine breeds. The answers to this questionnaire supported the presumptive diagnosis of NCL. The dog was euthanized by intravenous injection of sodium pentobarbital at 14 months of age at the owner's request. At necropsy, brain, eyes, muscle and blood were collected at the clinic in Florida and shipped immediately to the University of Missouri for further analysis. Slices of cerebral cortex and hind leg muscle were immediately frozen and shipped on dry ice, then stored at –80°C until used for further analysis.

The remainder of the brain and one eye were fixed immediately in neutral buffered formalin. All samples were collected with the full, informed consent of the dog's owners.

2.2 Nucleic Acid Isolation

Canine genomic DNA was prepared from white blood cell pellets obtained after lysis of red blood cells in 0.15M NH₄Cl as previously described [27]. Canine RNA was prepared from 5mg of brain tissue. RNA isolation was carried out using the SurePrep TrueTotal RNA Purification Kit from Fisher Bioreagents according to the manufacturer's instructions. Frozen tissue was disrupted by homogenization in a Roto-stator rotary homogenizer.

2.3 Microscopic analysis

Upon receipt in Missouri, the cornea, iris, lens and vitreous were removed from the eye, and the remainder of the globe was transferred to 170mM sodium cacodylate, pH 7.4 and incubated at room temperature for 4 hours with gentle agitation. The tissue was then stored at 4°C until further processing. Sections of the parietal lobe of the cerebral cortex and of the cerebellum were dissected from the brain. One piece of each brain area was washed and stored in cacodylate buffer for light microscopy, and another slice of each area was washed briefly in the same buffer and then transferred to a mixed aldehyde fixative for transmission electron microscopy (TEM) [32]. The samples for TEM were incubated in fixative with gentle agitation for three hours at room temperature and then stored in the fixative at 4°C until further processing. The samples stored in the cacodylate buffer were embedded and frozen in optimal cutting medium (Tissue-Tek). For the eye, samples included regions of the central, mid-peripheral and far-peripheral retina. Five micron sections of each tissue were cut with a cryostat and examined for the autofluorescent storage material characteristic of NCL [33]. For TEM, samples were post-fixed with osmium tetroxide, embedded in a epoxy resin, and 70–90 nm thick sections were cut and placed on copper grids. The sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1400 transmission electron microscope [32].

2.4 Nucleic acid amplification and sequencing

The coding regions and flanking DNA of all nine canine *PPT1* exons were amplified by polymerase chain reaction (PCR) using the primers shown in Table 1 with the Expand High Fidelity PCR System (Roche) according to the manufacturer's instructions. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced with a 3730xl DNA analyzer (Applied Biosystems, Foster City, California).

PCR primers 5'-GATCTGGCATGGGATGGGAG-3' (spanning the exon 1/exon 2 boundary) and 5'-TG TAGAATCCAAACCACTCAGAGTCC-3' (spanning the exon 7/exon 8 boundary) were used to amplify the region of the canine *PPT1* cDNA sequence (GenBank accession [NM_001010944](#)) surrounding the exon 5/exon 6 splice junction by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA from frozen brain tissue was used as template in the reaction and amplifications were carried out using a OneStep RT-PCR Kit (Qiagen) according to the manufacturer's instructions. Samples from the affected Dachshund and an English Setter with no documented history of NCL were used as templates. The amplicons were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. The RT-PCR products were purified and sequenced as described above.

2.5 Genotype Screening

Two sequence variants were identified in the *PPT1* gene of the affected dog, a 248 bp short interspersed nuclear element (SINE) inserted into intron 5 and a single base insertion in exon 8 (*PPT1* c.736_737insC). Primers 5'-CTGAAAAGGATGCAGAACTCGG-3' and 5'-GGCCTGGAATCACTGCTGGAG-3' were used to screen individual canine samples for the

248 bp SINE insertion by PCR amplification across the insert region. A Taqman allelic discrimination assay was used to genotype individual canine DNA samples at *PPT1* c. 736_737insC [34]. The PCR primer sequences were 5'-GATCATCTTCCTTCTTGCTCCTTCT-3' and 5'-TCTCCTGCAAGGGAATGGTTTC-3'. The probe sequences were 5'-VIC-CCACTTCTGTAGAATCCAA-MGB-3' for the wild-type allele and 5'-FAM-CACTTCTGTAGAGATCCAA-MGB-3' for the mutant allele. Samples were analyzed on a StepOne Plus Real-time PCR System (Applied Biosystems).

2.6 Enzyme Activity Assay

Frozen cerebral cortex gray matter from the affected Dachshund and one healthy homozygous normal Dachshund and two healthy English Setters were analyzed for PPT1 enzyme activity. All tissues were collected and stored under similar conditions. Researchers performing the assays were blinded with respect to the genotype and disease status of the dogs from which the samples were obtained. Tissues were homogenized in four volumes of buffer containing 50mM Tris-HCl, pH 7.0, 150mM NaCl, 1mM EDTA, 1µg/ml leupeptin, 1µg/ml pepstatin, and 0.2mM PMSF using a Brinkman polytron homogenizer at 4°C. Homogenates were centrifuged for 60 min at 100,000g. The supernatant was recovered and analyzed for protein concentration using a DC Protein Assay Kit (Bio-Rad). Palmitoyl protein thioesterase activity was assayed in duplicate using the artificial substrate, 4-methylumbelliferyl-6-thiopalmityl-β-D-glucopyranoside as previously described [35].

3. Results

3.1 Clinical observations

An intact male Dachshund was referred to the Veterinary Specialists of South Florida at 9 months of age after having exhibited a protracted period of progressive neurologic signs. In addition to the signs described below that were reported by the owners and primary care veterinarian, the neurologic exam also revealed kyphosis and stiffness of gait. The disease progressed over the following months to include uncontrolled rhythmic head movements, ataxia, and general weakness.

The abnormal signs observed by the owners starting prior to 9 months of age and progressing until the dog was euthanized included increased nervousness, decreased interactions with other dogs in the household, a severe loss in the ability to recognize or respond to commands or his name, a loss in the ability to recognize the primary owner or other people in the household, an increased sensitivity to loud noises, circling behavior, increasing inappropriate vocalization, loss of ability to climb stairs or other obstacles, tremors, loss of coordination, severe loss of vision, persistent head movements, and bumping into obstacles. Fundoscopic analysis by the primary care veterinarian at 7 months of age revealed diffuse retinal thinning and severe retinal vessel degeneration. By 8 months the dog appeared to be completely blind. The subject was tested for an NCL mutation of Dachshunds previously identified in the canine *TPP1* gene and the results were negative [25]. The dog was euthanized at 14 months of age due to the severity of the neurological signs.

3.2 Microscopic analysis

Microscopic examination of fixed tissues showed massive accumulation of autofluorescent storage material characteristic of NCL in neurons of the retina, cerebellum and cerebral cortex (Figs. 1 & 2). The central retina retained its normal layered structure, with autofluorescent inclusions present in the photoreceptor inner segments, the outer nuclear layer and to a lesser extent in the ganglion cell layer (Fig. 1B). The peripheral retina was greatly thinned with apparent complete loss of the photoreceptor cell layer and no recognizable layering typical of the normal retina. Autofluorescent storage material was present throughout the entire thickness

of the remaining peripheral retina (Fig. 1C). Autofluorescent storage material was abundant and widespread throughout the cerebral cortex and was not limited to a specific cell layer in this tissue (Fig. 2A and B). In the cerebellum cells filled with autofluorescent material occurred primarily in the granular layer, but could be found in all layers of this structure (Fig. 2C and D). Little or no autofluorescent material accumulation was observed in the Purkinje cells. Ultrastructural examination of the cerebral cortex and cerebellum indicated that the storage material was contained in membrane-bound inclusions and consisted largely of uniform granular-appearing material, typical of the GROD described in some human NCLs, primarily classical INCL (Fig. 3A) [20]. However, there was some heterogeneity in the storage bodies, with the contents of some inclusion bodies having a more complex ultrastructural appearance (Fig. 3B).

3.3 PPT1 resequencing

Resequencing of *PPT1* revealed two areas that differed from the canine reference sequence; a 248 SINE was inserted 17 bp downstream from the 5' end of intron 5 (Fig 4) and a single cytosine inserted 10 nucleotides from the 5' end of exon 8 generating a c.736_737insC mutation (Fig 5A). The insertion in exon 8 causes a frame shift which alters the predicted amino acid coding after Gly245, leading to a premature stop codon at position 276 in the altered protein (Fig 5B).

3.4 cDNA sequencing

Although the 248 bp SINE insertion lies completely within intron 5 of *PPT1*, its proximity to the 5' splice site led us to question whether it might disrupt splicing at the exon 5/exon 6 junction. Thus, RT-PCR was performed on RNA isolated from brain tissue using primers which generated a product that spanned exons 2 through 7. Template RNA from the affected dog produced a single product which migrated through agarose gel at the same rate as a product generated by RNA from an unrelated, homozygous normal, control Dachshund (data not shown). Sequencing of this amplicon confirmed normal splicing of consecutive exons in products from both the affected and control dog.

3.5 Allele Screening

We were able to obtain DNA from the subject's parents and paternal uncle. All three related dogs were heterozygous for the 248 bp SINE insertion and the c.736_737insC mutation. None of the three related dogs, all of whom are currently over 5 years of age, have displayed any signs of neurological abnormalities. We also screened DNA from unrelated Dachshunds for both sequence variants. Samples for these analyses were obtained from the University of Missouri Animal DNA Repository. Of the 127 additional Dachshunds screened, five were heterozygous for the 248 bp SINE insertion. The remaining 122 dogs were identical to the canine reference sequence at this polymorphic site. The c.736_737insC allele was not detected in any of the 127 unrelated Dachshunds. The only dogs found to possess the c.736_737insC allele were the propositus and his close genetic relations.

3.6 Enzyme Activity Analysis

The c.736_737insC allele causes a frame shift which results in a premature termination codon in the final exon of the gene. As a result, this mutation was predicted to encode a polypeptide with an altered and truncated N-terminal end which lacks both His289 and alters or eliminates key amino acids which contribute to the hydrophobic protein core. His289 and the hydrophobic core are required for thioesterase activity [41]. We predicted that these alterations would eliminate enzyme activity. To confirm this, we measured thioesterase activity in cerebral cortical tissue from the affected dog and three control dogs that were homozygous for the normal *PPT1* allele. Table 2 shows the results for these assays. The mean $\pm$ SD for enzyme

activity in the three control dogs was 5.5 ± 0.8 nmoles of hydrolyzed substrate/min/mg of total lysate protein, compared to an activity in the control dog of 0.16 nmoles/min/mg, which was 3% of the mean activity measured in the three control dogs. This value is virtually the same as that observed in assays of brain tissue from a PPT1 knockout mouse model with a deletion in exon 9, where the residual activity ($2.4 \pm 3.5\%$) is presumably attributable to other cellular thioesterases [29,36].

4. Discussion

Several reports of NCL in Dachshunds have been published in the past. In 1977 Cummings and de Lahunta described a Wire-haired Dachshund that developed a progressive neurodegenerative disease with onset at three years of age [37]. In 1980, Vandeveld and Fatzer described two long-haired Dachshunds which exhibited neurodegenerative signs at 5 and 7 years of age and were discovered to have autofluorescent inclusions in brain tissues [38]. Differences in the age of onset and clinical signs between these previous reports and the current case strongly suggest that the diseases described do not result from the same mutation. In 2006, we described a mutation of the canine *TPPI* gene in Long-haired Dachshunds which causes an early onset form of NCL [39]. This mutation was eliminated as the cause of NCL in the current case by genetic testing.

Both human and canine PPT1 proteins consist of 306 amino acids, including a signal sequence of 26 amino acids. Three amino acids (Ser115, Asp 233 and His289) comprise a catalytic triad and are essential for enzyme activity [40]. The protein contains a hydrophobic core which serves as a binding site for the fatty-acid modified substrates of the PPT1 enzyme. Disruption of this core also causes loss of enzyme activity [40]. To date, 48 mutations of *PPT1* have been identified in human NCL patients [18]. Mutations which affect one of the catalytic triad residues or disrupt the hydrophobic core of the protein cause the most severe, infantile, form of the disease [18]. The mutation reported here is a single base insertion in the coding sequence at nucleotide position c.736_737. This frame shift leads to loss of the catalytically active His289 residue and, based upon the previously published crystal structure, would cause loss or modification of a number of residues that contribute to the hydrophobic core of the protein [40]. These changes were predicted to completely eliminate PPT1 thioesterase activity and this prediction was confirmed through enzymatic activity assays. Although there was some remaining substrate hydrolysis in brain homogenate from the affected dog, this activity is likely to be the result of a second enzyme, PPT2, which is known to hydrolyze the assay's fluorogenic substrate at 4% of the rate of PPT1 and is abundant in brain tissues [36]. Using the same enzymatic assay employed in the present study, mouse brain tissue derived from *PPT1* knockout mice (which have no detectable PPT1 protein) shows 2.4% residual activity [29]. Brain lysate from human INCL patients with *PPT1* mutations often display similar levels of background activity in this assay [19]. Enzyme activity in brain tissue lysate from the affected dog was 3% of that found in brain tissue lysates from homozygous normal dogs, an activity level similar to both mice and humans with classical INCL mutations.

The distribution of lysosomal storage granules in the retina of the Dachshund with the *PPT1* mutation was significantly different from that which occurs in dogs with NCL resulting from mutations in other genes [25,27,28]. In other forms of canine NCL, the storage material is restricted primarily to the ganglion cell layer and in some cases the photoreceptor inner segments as well [39]. In *Ppt1* knockout mice, autofluorescent storage material accumulates primarily in the ganglion cell layer and inner nuclear layer, with minimal accumulation in the photoreceptor inner or outer segments [30,31]. In the current case, the storage material was widely distributed among all retinal layers. This more closely resembles human INCL, where autofluorescent material is observed in all neuronal retinal cell types [41]. The affected Dachshund displayed severe degeneration of the peripheral retina with massive accumulation

of storage material throughout the remaining layers. This differs from human INCL and other canine NCLs, where peripheral photoreceptors are often preserved even in later stages of the disease [25,27,28,41].

In the cerebral cortex, the distribution of cells containing autofluorescent storage material was widespread and uniform throughout the gray matter. In the cerebellum, the distribution of storage material was more specific, with accumulation occurring primarily in the granular layer. This differs from other canine forms of NCL where significant accumulation of autofluorescent material occurs in the Purkinje cells [25,42].

Although the ultrastructure of the storage material in the NCLs can be somewhat heterogeneous, even within the same individual, one type of storage material predominates in human INCL and in *Ppt1*-knockout mice [29,30,43]. In both cases the storage material consists primarily of GROD, whose contents exhibit a fine, uniform granular appearance. This was the predominant form of storage material in the propositus, although some granules with partial membranous structures were also observed in the brain. Similar heterogeneity has been noted previously in human INCL as well [1].

In addition to the c.736_737insC mutation, the affected Dachshund was also homozygous for a 248 bp SINE insertion located in intron 5 of the *PPT1* gene, which is unlikely to be related to the disease phenotype in this dog. Of 127 Dachshunds who were not closely related to the affected dog, 5 carried a single copy of the 248 bp SINE insertion. None of these dogs carried a copy of the c.736_737insC mutation. The affected dog's parents and paternal uncle each carried one copy of the c.736_737insC and the 248 bp SINE insertion alleles. These genotypes suggest that the disease-causing mutation originated on the 248 bp SINE insertion background. Given that this polymorphism is found in 3.9% of the Dachshunds in our DNA repository, the disease-causing mutation is likely to be rare in the general Dachshund population.

A canine model of INCL would be useful in evaluating clinical treatments for human NCL patients. PPT1 is a soluble enzyme that is known to traffic to lysosomes in peripheral cells by means of the mannose-6-phosphate receptor system [44]. As a result, PPT1 can be released into the extracellular milieu through recycling endosomes and taken up into the lysosomes of neighboring cells through mannose-6-phosphate receptor-containing endocytic vesicles [45]. In cell culture studies, it has been shown that exogenous PPT1 can be taken up from medium and can reverse the accumulation of autofluorescent lysosomal storage material in PPT1-deficient cells [44,46]. Gene therapy trials using an adeno-associated virus vector injected into the brains of *Ppt1*-knockout mice have also met with some success. These trials demonstrated that PPT1 enzyme activity was present even in cells not transformed by the gene replacement vector [47]. Although these experiments demonstrate proof of principle for INCL treatments, mice are likely to have limitations as models for human INCL therapies due to differences in brain volume and morphology between humans and mice. Distribution of exogenous protein throughout the brain is required for treatments depending on enzymatic cross-correction. As a result, the efficacy of any enzyme-based therapy for INCL may be influenced by the volume of tissue being treated and the anatomy of fluid-filled brain structures; factors which are significantly different between humans and mice. A large animal model, with greater similarities to human patients in brain volume, anatomy, cognitive abilities, and development would prove invaluable for evaluating INCL therapies. Unfortunately, the three carriers for the *PPT1* c.736_737insC mutation we have identified so far are not available for breeding. Two have been neutered and no sperm cells were detected in semen from the third dog. A test mating of this dog to a female Dachshund known to be fertile from previous breedings failed to result in a pregnancy. The existence or location of other potential relatives of the propositus are unknown. Efforts are currently under way to identify additional affected or carrier dogs in the

Miniature Dachshund population for use in establishing a research colony. Fibroblasts from one of the carrier dogs have been preserved for potential use in cloning.

Acknowledgments

Funds to support the work reported here were provided by the American Kennel Club Canine Health Foundation (grant 732, M.L.K. and grant 762, G.S.J.), the Batten Disease Support and Research Association (M.L.K.), an unrestricted grant from Research to Prevent Blindness, Inc (M.L.K.) and NIH grant R37NS036867 (S.L.H). Our thanks to the owners of the dog described in this study for donating the tissues and to Dr. Lisardo Martinez from West Bird Animal Hospital who originally examined the dog and referred him to us. We would like to thank Dr. Dietrich Volkmann of the University of Missouri Department of Veterinary Medicine and Surgery for assistance with canine fertility evaluations and breeding and the staff of the University of Missouri Electron Microscopy Core Facility for their expert assistance.

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Figure 1.

Fluorescence micrographs of cross sections of the central retinas from an age-matched normal dog (A) and the affected Dachshund (B). The bright band of autofluorescence in the normal dog originates from the retinal pigment epithelium (RPE), and occurs normally in most mammals, including the affected dog. The yellow autofluorescence in the neural retina above the RPE is a specific marker for NCL (B). The nonfluorescent band between the RPE and neural retina in (B) represents the photoreceptor outer segments. Most of the autofluorescent material in the central retina occurs in the photoreceptor inner segments and outer nuclear layers. Less autofluorescence is present in the ganglion cell layer. The peripheral retina of the affected dog (C) was detached from the RPE and greatly thinned with loss of the layered organization. The autofluorescent material accumulation in the peripheral retina occurs throughout the retinal thickness. Bar in (C) indicates the magnification of all 3 micrographs.

Figure 2.

Fluorescence micrographs of brain tissue sections from an age-matched normal dog (A and C) and the affected Dachshund (B and D). Massive amounts of autofluorescent lysosomal storage material were observed in cerebral cortex (B) and primarily in the granular (g) layer of the cerebellum (D) in the affected dog. Lesser amounts of storage material were present in the Purkinje cells (p) and molecular (m) layers. Levels of autofluorescent material in similar brain regions from the normal dog were nearly undetectable. Bar in (A) indicates magnification in all 4 micrographs.

Figure 3.

Transmission electron micrographs of storage bodies (arrows) from the cerebral cortex (A) and cerebellum (B) from the affected dog. The contents of most of the storage bodies had a fine granular appearance typical of GROD in humans with INCL. However some of the storage bodies contained heterogeneous clumped aggregates of material, some of which exhibited partial membranous substructure (arrows in B). Both types of storage bodies were found in the cerebral cortex and cerebellum. Bar in (A) indicates the magnification of both micrographs.

WT Allele	2872351	AACAAAGCTA	TTCAAGAACG	gtacgtatgg	ttggctgaaa	aggatgcaga
Insertion Allele		AACAAAGCTA	TTCAAGAACG	gtacgtatgg	ttggctgaaa	aggatgcaga
	2872402	act-----	-----	-----	-----	-----
		:~::~:	:~::~:	:~::~:	:~::~:	:~::~:
		<u>actcggggat</u>	ccctgggttg	cgcagcggtt	tagcgcctgc	ctttggccca
	2872404	-----	-----	-----	-----	-----
		:~::~:	:~::~:	:~::~:	:~::~:	:~::~:
		gggcgcgatc	ctggagaccc	gggatcgaat	cccacgtcgg	gctcccagtg
	2872404	-----	-----	-----	-----	-----
		:~::~:	:~::~:	:~::~:	:~::~:	:~::~:
		catggagcct	gctctctcct	ctgcctatgt	ctctgcctct	ctctctctca
	2872404	-----	-----	-----	-----	-----
		:~::~:	:~::~:	:~::~:	:~::~:	:~::~:
		ctgtgtgcct	atcataaata	aataaaatta	aattaaaaaa	tttaaaaaaa
	2872404	-----	-----	-----	-----gtcac	tgtgtgaaaa
		:~::~:	:~::~:	:~::~:	:~::~:	
		aaaaaaaaa	aaaaaaaaa	<u>aaaggatgca</u>	<u>gaactgtcac</u>	tgtgtgaaaa
	2872419	tatgctgact	ttctctccag	cagtgattcc	aggccacgga	tactcacatg
		tatgctgact	ttctctccag	cagtgattcc	aggccacgga	tactcacatg

Figure 4.

Partial DNA sequence alignment of wild type canine *PPT1* and sequence from the NCL-affected Dachshund, including the 248 bp SINE insertion in intron 5. Numbering corresponds to the canine chromosome 15 genomic contig sequence (GenBank accession [NW_876259.1](#)). Exon sequences are in uppercase, with intron sequences in lowercase. The 248 bp SINE insertion includes 16 bases duplicated from the 5' end of intron 5 in the native *PPT1* sequence (underlined).

Figure 5.
 (A) Sequence traces of exon 8 showing the presence of the c.736_737insC mutation in an affected and carrier dog. Sequences from the carrier and affected dog are identical with the normal dog for the first 10 bases (up to the black line), after which they diverge. The carrier trace is composed of overlapping sequences identical to the normal and affected trace. (B) Amino acid sequence comparing the wild type PPT1 sequence (GenBank accession [CAI44937](#)) with the predicted amino acid sequence from the affected dog. The frameshift mutation results in missense mutations starting at amino acid position 246 and a stop codon which truncates the protein at amino acid position 276. Amino acids comprising the PPT1 catalytic triad are boxed.

Table 1Sequence of primers used for amplification and sequencing of *PPT1*¹

Exon	Sequence	Product
1	5 - CCGGAAGGCGAGGCCGAAC -3	295bp
1	5 - AACCAGGTCGGGCGAGCG -3	
2	5 - GCCAAAGGCAGATACTCAACCAT -3	265bp
2	5 - TCAGCAAACCTTCAAAGGGTCAA -3	
3	5 - CTTGAGTAGTGGGAGC -3	307bp
3	5 - TTCGAGGAATGGATC -3	
4	5 - TAATCTGGGAAGTTGGTTGTTGTGATG -3	278bp
4	5 - ACTTTGTTATAAGCTGGGAAAACAG -3	
5	5 - TATTTGATACACAACCTATTGAAGGG -3	185bp
5	5 - GCATCCTTTTCAGCCAACCATAC -3	
5	5 - CAACTATTGAAGGGATGAATGGATT -3	656bp
5	5 - AGGCTTGGTACTTTCTAAAGGCT -3	
6	5 - GCTGCCTTCACTCGTTC -3	233bp
6	5 - TGCCTCCCAGTCTGTCC -3	
7	5 - GCTGGGCAGGCTTTA -3	217bp
7	5 - GCAGGTGGCTCTATCTTA -3	
8	5 - CGGCCAGTCCTAGCTTAAC -3	265bp
8	5 - CTCACCCGTAGCAGGCACA -3	
9	5 - CACTTTTGAGCATGTATCCCA -3	366bp
9	5 - GGGCATGTTAGGTGATAACTA -3	

¹The annealing temperature for all reactions was 58 °C

Table 2**PPT1 Enzyme Activity Assay Results**

Subject ¹	1 st Replicate nmol/min/mg	2 nd Replicate nmol/min/mg	Mean nmol/min/mg ²
Control 1	5.5	5.6	5.55
Control 2	4.6	4.8	4.70
Control 3	6.0	6.6	6.30
Affected	0.15	0.18	0.16

¹ PPT1 enzyme activity levels were assayed in brain tissue lysate from three homozygous normal control dogs and the affected dog.

² Activity level values represent nanomoles of substrate (4-methylumbelliferyl-6-thiopalmityl- β -D-glucopyranoside) hydrolyzed per minute per milligram of total lysate protein.