

THREE MUTATIONS THAT CAUSE DIFFERENT FORMS OF
CANINE NEURONAL CEROID LIPOFUSCINOSIS

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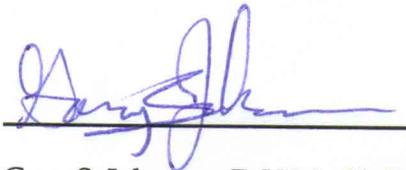
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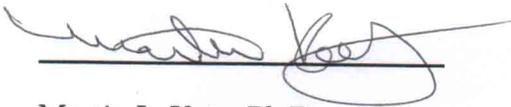
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THREE MUTATIONS THAT CAUSE DIFFERENT FORMS OF CANINE NEURONAL CEROID LIPOFUSCINOSIS

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ABSTRACT

Neuronal ceroid-lipofuscinosis (NCL), also known as Battens disease, is really a group of inherited neurodegenerative diseases. A common feature of the ceroid lipofuscinoses is the deposition of autofluorescent cytoplasmic storage material in cells in the brain, retina, and many other tissues. The major symptoms are mental retardation, visual failure, loose of motor skills, seizures, and eventually premature death. In the European countries and USA, the disease affects one in 12,500 to 100,000 people. Usually children appear to be healthy at the birth and develop normally until onset of disease.

Canine NCLs have been reported in a variety of breeds where they are important as veterinary diseases and as potential models for the human NCLs. We have discovered that a missense mutation in the *CLN8* gene causes NCL in English Setters, a missense mutation in the *CTSD* gene causes American Bulldog NCL, and frame shift mutation in the *CLN2* gene causes Dachshund NCL.

Chapter I: Introduction

Neuronal ceroid-lipofuscinosis (NCL), also known as Battens disease, is really a group of inherited neurodegenerative diseases. A common feature of the ceroid lipofuscinoses is the deposition of autofluorescent cytoplasmic storage material in cells in the brain, retina, and many other tissues. The major symptoms are mental retardation, visual failure, loose of motor skills, seizures, and eventually premature death. In the European countries and USA, the disease affects one in 12,500 to 100,000 people. Usually children appear to be healthy at the birth and develop normally until onset of disease [1].

Currently, there is no effective medication. Diagnosis is based on clinical signs and confirmed by fluorescence microscopy and electron microscopy of post mortem brain sections. However, these techniques cannot identify pre-clinical patients or heterozygous carriers. Genetic diagnosis can be used to screen for common disease alleles [2, 3].

Several NCLs have been recognized in people and various domestic and laboratory animals. Canine NCLs have been reported in a variety of breeds

where they are important as veterinary diseases and as potential models for the human NCLs. We have been searching for loci responsible for NCL in several dog breeds and have discovered that a missense mutation in the *CLN8* gene causes NCL in English Setters [4], a missense mutation in the *CTSD* gene causes American Bulldog NCL [5], and frame shift mutation in the *CLN2* gene causes Dachshund NCL[6]. Chapters 2, 3, and 4 of this thesis contain the text of the publications reporting these findings.

Chapter II: Literature review of neuronal ceroid-lipofuscinosis

Classification of NCLs

Neuronal ceroid lipofuscinosis was first reported in the early 19th century in Norway based on observations of progressive blindness, epilepsy, cognitive decline, and ataxia [7]. It was characterized as Juvenile NCL. A century later, patients with a similar intraneuronal storage disorder but with late infantile onset and adult onset were reported [8]. These NCLs were initially thought to be a form of gangliosidosis. However, the histochemical characteristics of the storage material distinguished these diseases from gangliosidoses and they were

termed as “ceroid-lipofuscinoses” [9]. Later, an infantile NCL was reported [10-12].

The NCLs have been subclassified based on the age of onset and ultrastructure of the storage material. The early literature recognized four main forms: infantile (INCL or CLN1), late infantile (LINCL or CLN2), juvenile (JNCL or CLN3), and adult (ANCL or CLN4). Since then, several variant cases have been reported. Molecular genetic analyses of the NCLs in the 1990’s lead to the discovery of six genes (*PPT1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, and *CLN8*) that cause NCL in people [13-18]. In addition, naturally occurring NCL of sheep is caused by a mutation in the cathepsin D gene (*CTSD*) [19] and *PPT2*, *CLCN3*, *CLCN7*, and *CTSF* knockout mice showed an NCL-like disease [20-23]. Genetic analysis has shown that different mutations in the same gene can result in different phenotypes (i.e., Northan epilepsy and Turkish form) [15, 24].

Infantile Neuronal Ceroid Lipofuscinosis (INCL)

Mutations in the palmitoyl protein thioesterase 1 gene (*PPT1*) can cause INCL [25]. Among the Finnish population, 95% of patients have an A364T

transition which changes Arginine-122 to Tryptophan. Later genetic analyses identified 21 different mutations in *PPT1* from various ethnic backgrounds [25]. The *PPT1* mutations in INCL patients cause almost complete loss of palmitoyl protein thioesterase 1 activity; however, there were some recently discovered *PPT1* mutations that produce later onset disease, apparently because the mutations only partially destroyed enzyme activity [26]. The characteristic histological feature of *PPT1* mutations is the accumulation of cytoplasmic storage bodies in neuronal cells and other cells. Under the electron microscope, the storage bodies appeared as membrane-bound conglomerates of round electron-dense globules with a finely granular internal ultrastructure known as granular osmiophilic deposits (GROD) [27]. The major protein components of the storage granules are saposins A and D, also called as sphingolipid activator proteins (SAPs) [28]. The SAPs function by facilitating the access of hydrolytic enzymes to their lipophilic substrates [29]. It is likely that the SAPs deposition in storage bodies is a response to the sphingolipids that are also accumulating in the storage granules.

Palmitoyl protein thioesterase 1 is the lysosomal glycoprotein that catalyzes the removal of palmitate attached to proteins through a thioester bond. The precise physiologic role of palmitoyl protein thioesterase 1 has not been established and it is not clear how *PPT1* mutations relate to early neuronal death. Over expression of *PPT1* prevented neuronal cell apoptosis and a palmitoyl protein thioesterase 1 inhibitor induced apoptosis in Ras-Akt-caspase pathway [30, 31]. Therefore, *PPT1* may play an important role in cell maintenance and survival.

Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL)

Mutations in the *CLN2* cause LINCL. Twenty five different missense, nonsense, deletion, insertion and splicing mutations had been reported by 1997 [17]. Usually, the first symptom is seizures occurring at 2 to 4 years of age. Neurodegeneration progresses with blindness occurring at 5 or 6 years of age and death at mid childhood [32, 33].

The *CLN2* gene encodes tripeptidyl peptidase I which removes tripeptides from the amino-terminus of peptides [34]. In electron micrographs,

the storage bodies from LINCL patients are filled with curvilinear forms. The storage bodies are composed of proteins and lipids and are strongly immunoreactive with antibodies to subunit C of mitochondrial ATP synthase [35]. The subunit C accumulation has also been reported in other forms of NCL like Finnish variant LINCL (*CLN5* deficiency) [2] and the Indian/Costa Rican variant form (*CLN6* deficiency) [14]; however, subunit C does not accumulate in INCL [36]. A pulse/chase study showed that subunit C synthesis was normal but that subunit C degradation was delayed in LINCL [37]. It seems likely that there is no post translational modification of subunit C, because subunit C isolated from LINCL patients was degraded by a normal lysosomal extract [37]. Treatment of a normal lysosomal extracts with anti-tripeptidyl peptidase I antibodies reduced subunit C degradation compared to the degradation by an untreated control extract [38]. Therefore, it appears that tripeptidyl peptidase I is directly or indirectly required for normal subunit C turnover. It is not known if the subunit C accumulation contributes to the neuronal cell death in LINCL patients.

Juvenile Neuronal Ceroid Lipofuscinosis (JNCL)

Juvenile NCL, the most common form of the human NCLs, is caused by mutations in *CLN3* [13]. The first symptom, progressive visual failure, occurs at 4 to 7 years of age. Many patients show the signs of parkinsonism by mid-teens [32, 39], and patients usually die in their 20's [40].

Fingerprint forms are typically seen in electron micrographs of cytoplasmic storage bodies from JNCL patients [41]. The storage bodies contain SAPs A and D as major components [29]. The subunit C of mitochondrial ATP synthase is also present, but this protein is much less prominent in JNCL storage bodies compared to LINCL storage bodies [29]. The typical ultrastructural appearance in the JNCL storage bodies is a fingerprint form [41].

The function of *CLN3* protein is not known, but computer predictions based on amino acid sequences suggest that the protein is an integral membrane protein with multiple transmembrane-spanning regions [42]. *CLN3* amino acid sequence is evolutionarily highly conserved, and homologous genes to *CLN3* have been identified in various species [43-46]. In yeast, *BTN1* is a homolog of human *CLN3*. Yeast which lacked *BTN1* protein did not acidify a vacuole which is

analogous to the lysosome [47]. The CLN3 protein is suspected to play an important role in cell metabolism such as organelle fusion/transport or disposal of organelle membranes [48].

Adult Neuronal Ceroid Lipofuscinosis (ANCL)

ANCL is relatively rare among the human NCLs. The average age at clinical onset is 30 years and the range is 11 to 50 years [49]. ANCL has been subclassified into two clinical phenotypes characterized by progressive myoclonus epilepsy (type A) or dementia with motor disturbances (type B). Some type A cases had photosensitivity, while type B commonly showed dyskinesia [49, 50]. The other clinical features of ANCL are similar to those of other NCLs except visual symptoms are seldom present [51]. Autofluorescent cytoplasmic storage bodies are characteristic of ANCL. Electron microscopy has revealed fingerprint forms, rectilinear forms, and GROD [52, 53]. The storage bodies were more prominent in neuronal cells compared to the cells from other tissues [51].

Usually NCLs segregate in families in an autosomal recessive inheritance pattern; however, several cases ANCLs were reported to be autosomal dominant diseases [50, 52-54]. Therefore, the causes of ANCL are likely to be genetically heterogeneous [49].

Variant forms of NCLS

A variant form of LINCL known as the Finnish variant is caused by *CLN5* mutations [3, 16]. This form is pathologically and clinically distinctive from the usual LINCL. Typically, the first clinical sign is motor clumsiness which occurs at 4.5 to 6 years of age. This is followed by progressive visual failure and, eventually, blindness, seizures, and finally death at 14 to 32 years of age [55]. The storage bodies in the Finnish variant LINCL have various appearances; however rectilinear forms are most common in cerebral neurons [27]. The major component of the storage body is subunit C of mitochondrial ATP synthase and SAPs [56]. Several mutations in the *CLN5* gene have been reported [16]. The 2-bp deletion in exon 4 is the most common mutation; it has been identified in over 90% of Finnish LINCL patients [3, 16].

Another variant form, called the early juvenile variant, is caused by mutations in *CLN6* [57]. This variant mainly occurs in southern Europe [58]. The clinical features of the early juvenile variant resemble those of classical LINCL; however, one third of the patients showed a slightly later onset [55]. The *CLN6* gene was identified by positional cloning [14]. It encodes a novel protein which contains seven predicted transmembrane domains.

The mutations in *CLN8* cause either “northern epilepsy” or the Turkish variant of LINCL. Northern epilepsy is also known as progressive epilepsy with mental retardation and has only been recognized in Finland [59-62]. The clinical onset of northern epilepsy is between 5 and 10 years of age and the first symptoms are generalized tonic-chronic seizures. Mental retardation is apparent 2 to 5 years after the onset of seizures. The ultrastructure of the storage body material shows mainly curvilinear forms and, occasionally, granular structures. Subunit C of mitochondrial ATP synthase is the major component of storage body [59].

The Turkish variant of LINCL is allelic to northern epilepsy, but the clinical phenotype is quite distinctive. The clinical symptoms of the Turkish

variant mostly overlap with those of classical LINCL. The main phenotypic difference between LINCL and the Turkish variant is that fingerprint forms predominate in electron micrograms of storage bodies from the Turkish variant whereas curvilinear forms are seen in classical LINCL electron micrograms [63].

The *CLN8* gene encodes a predicted transmembrane protein. Immunofluorescent microscopy showed that the CLN8 protein is located in the endoplasmic reticulum. Partial localization to the endoplasmic reticulum-Golgi intermediate compartment was also recognized. Therefore, the CLN8 protein is suspected as an ER resident protein that recycles between endoplasmic reticulum and endoplasmic reticulum-Golgi intermediate compartment [64].

Animal models

Canine NCLs are recognized in many breeds. Some breeds (Tibetan Terrier, English Setter, and Border Collie) have been proposed as animal model for human NCLs [65-67]. Canine NCLs are still rare disease as human NCLs. But in some breeds, NCL is considered a major problem, because of purebreed breeding patterns [68]. Any breed is potentially at risk. Therefore, the canine NCLs are not only important as animal models for human NCLs but also as veterinary diseases. So far, canine NCLs have been recognized in at least 19 breeds. Each breed has a characteristic form of NCL. The remainder of this chapter contains descriptions of previously reported canine NCLs.

Canine Neuronal Ceroid Lipofuscinosis

American Bulldogs

The age of onset in American Bulldog NCL was 0.9 to 3 years of age. The initial symptom was abnormal gait. Affected dogs showed uncoordinated movement in the pelvic limbs. As the disease progresses, symptoms the dogs developed a wide-based stance, first in the rear and eventually in all four limbs.

Next the dogs exhibit progressive ataxia and hypermetria. Muscle twitching might also occur, especially during sleep. In the later stages, conscious proprioception reactions were lost in all four limbs and the dogs are unable to rise up from the recumbent position without assistance. No visual abnormalities and seizures were recognized. Typically, the affected dogs were euthanized between 3.5 and 5.5 years of age [69].

Pathologically, no brain atrophy was recognized. Nonetheless, the surface of the brain was brownish and many storage materials were found in cerebral cortex, brainstem, cerebellum (especially in the Purkinje cells) and retina (especially the retinal ganglion cells). Electron microscopy showed the storage bodies containing a number of somewhat spherical aggregates within a granular field [69]. Disease is inherited as an autosomal recessive inheritance. We have discovered that a mutation in the cathepsin D gene causes American Bulldog NCL as indicated in chapter IV.

American Staffordshire Terrier and American Pit Bull Terrier

American Staffordshire Terriers and American Pit Bull Terriers with NCL were similar both clinically and pathologically [70]. The age of onset was between 2 and 5 years of age with gait abnormality, generalized cerebellar ataxia with hypermetria, stiffness, and loss of balance. Patients exhibited slowly progressive cerebellar-vestibular signs. They were euthanized between ages of 4 to 8 years. Neither behavior abnormality nor visual impairment was recognized [70].

At necropsy, the significant abnormalities were found only in restricted regions of the brain such as various degrees of atrophy of the cerebellum, inconsistent unilateral enlargement of lateral ventricles and inconsistent mild thalamic atrophy. Degradations occurred in Purkinje cells and certain thalamic nuclei associated with a lysosomal deposition. This is the first case of thalamic degeneration in NCLs. Purkinje cells and certain thalamic neurons showed membrane bound storage bodies in the cytoplasm [70]. Under the electron microscope, the storage bodies had a lamellar profile. The storage bodies were autofluorescent, consistent with a diagnosis of NCL. However, storage bodies in

NCLs are usually widespread throughout brain. These cases only have storage bodies in Purkinje cells and certain thalamic neurons. And also, thalamic neuronal degeneration associated with lipofuscin deposit was reported in human polyglutamine disease. Therefore, these cases might be caused by combination of NCL and polyglutamine disease [70]. Because the disease in these breeds differs from the other NCLs in that the storage material only accumulates in limited brain regions, it may not be appropriate to classify the American Staffordshire Terrier and American Pit Bull Terrier disorders as NCLs.

Australian cattle dog

Only two cases of littermates have been reported. The first symptoms were observed at around 1 year of age. The dog bumped its head into a wall and walked off a pier into a lake, suggesting visual dysfunction. As the symptoms progressed, the dog became less coordinated and showed fear-like reactions to strange noises and visitors. At the 26 months of age, the dog became unresponsive to the owner's commands and affection [71].

Neurological examination revealed an abnormal gait and circling, a wide-based stance, and bumping into objects suggesting blindness. However, no abnormalities were observed during ophthalmoscope examination. Also, truncal ataxia was recognized. The dog still had responses to auditory and tactile stimuli, but reactions were slightly demented. Dog was euthanized at 26 months of age [71].

Gross brain lesions were those of hydrocephalus with moderate dilation of lateral ventricle and atrophy of cerebrum and cerebellum. Histological examination showed storage body deposition in most of the neurons in brain and spinal code. Most of neurons showed abnormal morphology because of storage bodies in cytoplasm. The storage bodies were also recognized in a wide range of mesenchymal and epithelial cells in several visceral tissues such as kidney, urinary bladder, liver, retina, and adrenal glands. The ultrastructure of storage body was membrane-bound fingerprint and curvilinear structures. Autosomal recessive inheritance was suspected in Australian cattle dog NCL because of two littermates developed symptoms. However, additional analyses will be required

to confirm this putative mode of inheritance. The gene containing the causative mutation is unknown [71].

Border Collies

Border Collie NCL was widespread in Australia, because of the common use of a few champion dogs that carried the disease-causing allele. Symptoms are observed by owners at around 15 months of age and affected dogs seldom survive past 28 months of age [72].

The first symptom is usually hyperactivity with compulsive running or aimless wandering, and later continuous barking, agitation, and violence appear. The behavioral abnormality is initially episodic, but gets more frequent and severe as the disease progresses. Dogs may also show snapping at nonexistent objects, inappropriate startle responses, and biting and growling at inanimate objects. These actions may indicate visual and auditory hallucinations. Most affected dogs lose their ability to participate in everyday activities, such as loss of learned behavior (i.e.; house training), failing to respond to the owner's voice, episodic leg jerking, jaw champing and grinding of teeth, and difficulty in eating

and drinking. Seizures have been reported in some cases. Blindness and some degree of visual failure develop at the later stages of the disease [24, 72].

Ultrastructural examination of the retinal tissue showed granule accumulation, but no abnormalities were recognized under the fundoscopy. Most of the neurons and glial cells in the central nervous systems had cytoplasmic inclusions especially in the roof nuclei and Purkinje cells. In addition, cytoplasmic inclusions were found in many visceral tissues. The ultrastructure of the storage bodies included fingerprint and curvilinear forms [72]. The major component of storage body was subunit C of mitochondrial ATP synthetase [72]. The disease is an autosomal recessive trait. The linkage and sequence analysis revealed a CLN5 mutation causes Border Collie NCL [68].

Chihuahuas

Only single case has been reported so far. The first symptoms appear at 13 to 21 months of age with visual dysfunction and a slight reduction of the sense of smell. After that, the dog showed abnormal gait and behavior such as aggressiveness. Under neurological and ophthalmologic examinations, the dog

showed no abnormality except loss of pupillary reaction to the light. The dog was euthanized at 2 years of age [73].

On the gross examination, skull expansion was apparent. And, brain was remarkably dilated at the lateral and third ventricles. There were no microscopic abnormalities in the visceral organs. Much neuronal swelling was found in central nervous tissues. Neurons contained storage bodies that were most apparent in the hippocampus, thalamus, and Purkinje cells of the cerebellum. Retinal neurons also swelled and contained storage bodies. However, no storage bodies were found outside the central nervous tissues. The storage bodies had a dense lamellar ultrastructure. GFAP immunohistochemistry showed that the cerebellum had Bergmann gliosis in the molecular layer. The causative gene mutation is unknown [73].

Cocker Spaniels

Seven cases of Cocker Spaniel NCL have been reported. The age of onset is one and a half to 6 years of age. It starts with abnormal gait and lack of coordination, especially in the hind limb. Postural reactions and proprioception

deteriorate. Some affected dogs exhibited aggression, seizures, or blindness [74, 75].

The hallmark of Cocker Spaniel NCL is the heavy deposition of yellow-brown pigmentations that occurs in the smooth muscles of the gastrointestinal tract, a condition known as brown bowel syndrome [74]. This pigmentation was widely spread between muscle fibers, uterine muscle, the bladder and small arteries. Deposition of yellow-brown granules was also recognized in some neurons of brain and spinal code. Depositions were mainly recognized in neurons of the spinal code and basal bodies of cerebellum, with lighter deposits in the cerebral cortex, brain stem, and Purkinje cells. The ultrastructure of storage body was membranous laminate in form. An autosomal recessive inheritance is suspected. The gene mutation that causes the disease is unknown [76].

Dachshunds

Two isolated reports of Dachshunds NCLs were published in the past. In 1977, Cunmmings et al [77] reported wirehaired Dachshund neurodegeneration

which started with hind limbs abnormality at three years of age. The disease was slowly progressive, but retinal function was preserved. Pathologic examination revealed cerebellar atrophy, yellow discoloration of cerebellar nuclei and the pontine nuclear area, and enlargement of lateral and fourth ventricles. Cerebellar neurons and macrophages contained autofluorescent storage bodies in the cytoplasm which formed zebra bodies. In 1980, Vandeveldel et al [78] reported two longhaired Dachshunds with neurodegeneration with mental deterioration at five and seven years of age. Autofluorescent storage bodies were found in neurons of brains, but fingerprint forms were common, zebra forms were rare.

Recently, veterinarians in private practice provided records from two longhaired Dachshunds littermates. The first signs of neurodegeneration appeared at seven to nine months olds of age, and the dogs died at 1 year of age. The cells in central nervous systems contained autofluorescent storage bodies with curvilinear ultrastructures that closely resembled storage bodies in human LINCL. Two littermates developed disease suggested that the disease has an autosomal recessive inheritance. We detected a *CLN2* gene mutation causes this disease. (The details are in chapter V)

Dalmatians

Within 15 practicing deliberate inbreeding affected dogs, most of them first exhibited visual impairment around 6 months of age. After that, one fourth of the affected dogs showed behavioral abnormalities such as aggressiveness towards approaching individuals, running erratically and eventually biting each other. All affected dogs showed seizures around 15 months of age. There were also signs of self-mutilation and bruxism. At the 20 months of age, affected dogs showed ataxia, tremor, and seizures. By 22 months of age, dogs showed staggering and bumping into the objects. No abnormality was found by fundoscopic examinations. Usually, symptoms are severe after 20 months, but in some cases dogs can live 7 or 8 years of age [79, 80].

Upon pathological examination, the brain weight was reduced to about 65% of normal. And, granular material depositions were found in the neuronal perikarya of the cerebral cortex, the hippocampus, the basal ganglia, and brainstem. Purkinje cell and granular cell loss were recognized in the cerebellum. Retinal ganglion cells showed enlargement because of storage body deposition, but photoreceptor cells were still intact. Storage body accumulation was shown

ubiquitously in viscera. The ultrastructure of storage bodies was varied and granular, curvilinear, fingerprint, and membranous-lamellar, forms were recognized. There were no tissue specific forms. The inheritance pattern was not clear; however, autosomal recessive inheritance was suspected. The causative gene mutation is unknown [79, 80].

English Setters

From the 1950's, English Setters were studied as an animal model for human NCL. The age of onset is 14 to 18 months of age. The initial symptoms were vision reduction and mental dullness. Affected dogs showed progressive mental deterioration and begun to stagger with stiffened limbs. Seizures appeared at 17 to 24 months of age. Affected dogs can't survive over 27 months of age [65].

Mild brain atrophy was found at the age of onset. Toward the end of the disease, at 20 months of age, the brains show gross atrophy (60 to 70% of normal weight). Most of the neurons in the cerebrums, cerebellums, and spinal codes have cytoplasmic storage bodies. The storage bodies don't make the cell volume

increase, but rather replace normal intracellular constituents. In the later stages, storage bodies were also found in viscera; but, no gross abnormalities were found. The ultrastructure of the storage bodies varied and fingerprint, curvilinear, and granular osmiophilic forms were seen [65]. The disease exhibits autosomal recessive inheritance. We discovered a mutation in the *CLN8* gene causes this disease. (Details in chapter III)

Japanese Retrievers

A three year old dog showed sudden polydipsia and repeated vomiting. The dog died that same day. The conglomerates of granules were observed in neurons, particularly in the cerebral cortex. Under the electron microscopy, membrane-bounded granules were shown in the cytoplasm of neurons, astroglia, macrophages, and smooth muscle [81].

The cause of death was suspected to be tubulonephrosis caused by a nutritional defect, and not intractable neuronal symptoms. This NCL appears to be different from the NCL in other breeds [81].

Labrador Retrievers

The onset was at 8 months of age. The initial symptom was progressive partial seizure activity, characterized by facial and ear twitching while 11 months. Symptoms progressed but were limited to the nervous systems. Symptoms included hypermetric gait, truncal ataxia, and tetraparesis. Later, affected dogs showed pharyngeal dysphagia and generalized tonic-clonic seizure. No visual abnormality and brain atrophy were recognized [82].

Pathologically, granular material depositions appeared in the cerebral cortex, and hippocampus. Satellitosis was recognized in the midbrain and cerebral cortex, indicating neuronal distraction occurred or pre-distra horls. The mode of inheritance and cause of this disease are currently unknown.

Miniature Schnauzers

Two cases of Miniature Schnauzers NCL have been reported. Onset is at 2 to 4 years of age. The disease starts with visual impairment. At the age of 3 to 4 years, visual symptoms progressed quickly and lead to blindness. Other neurological signs also developed including confusion, depression, aimless

wandering, aggression toward the owner or other dogs, and loss of memory.

Affected dogs are usually euthanized at this age [83].

Pathological study showed severe photoreceptor degradation, and extensive accumulation of storage bodies in the central nervous tissues, retina, and liver. The storage bodies appeared as granular osmiophilic depositions in electron micrographs. The main storage body components were SAPs, and no subunit C of mitochondrial ATP synthase was detected. Autosomal recessive inheritance was suspected; but, the gene containing the mutation remains unknown [84].

Polish Lowland Sheepdogs (PONS)

The two cases of PONS NCL have been reported. And additional unpublished cases have been brought to our attention. The age of onset was 0.5 to 4.5 years of age. Affected dogs showed a mental change and appeared to be unhappy, anxious, irritable, and aggressive. A progressive gait abnormality was recognized, especially in the pelvic limbs. Abnormal pupillary light reflexes were

observed. Skin problems were a common symptom in affected dogs. The affected dogs were euthanized at 1.5 to 8 years of age [85].

Pathologically, brains from affected dogs had light brown discoloration. The accumulation of cytoplasmic storage bodies were observed in various neurons, and also in the lymph node, liver, kidney, and skin. The ultrastructure of the storage bodies was membrane-bound whorls and fingerprint forms. Autosomal recessive inheritance is suspected and the causative gene is unknown [86].

Salukis

NCL was reported in two related litters of Salukis. Symptoms, which appeared at 1 year of age, included hyperesthesia and blindness. On clinical examination, the dogs were uncoordinated and exhibited swimming movements associated with paresis of neck and limb muscles. The dogs could not stand even with assistance. However, pain or remarkable visual impairment was not recognized. No other clinical abnormality was recognized [87].

No gross lesion was found; but histopathologically the large neurons in the cerebrum, thalamus, hippocampus, cerebellum, medulla, and retina had granular pigment depositions in the cytoplasm. These deposits were also recognized in small neurons and glia. Many neurons were retained but some Purkinje cells were lost. Under the electron microscope, granular pigment depositions formed whorles or fingerprints surrounded by a single membrane. The occurrence of the disease in siblings suggested an autosomal recessive mode of inheritance. The causative gene mutation is not known [87].

Tibetan Terriers

Tibetan Terriers NCL has the characteristics of a slowly devolving visual abnormality. The first clinical sign was nyctalopia noted from 2 to 3 years of age. Later, behavior changes were recognized around 4 to 6 years of age, such as loss of house training, confusion, fearfulness, occasional unpredictable aggressiveness, and poor appetite. At 7 to 10 years of age, ataxia occurred and responses to visual, auditory, or tactile stimuli became dull. Affected dogs are usually euthanized around this age [66].

Mild brain atrophy becomes apparent only late in the course of the disease [66]. Under the electron microscope, osmiophilic depositions were recognized in retinal inner nuclear cells and ganglion cells at 8 weeks to 6 months of age. By 3 years of age, cellular loss occurred in the inner retinal layers in both the tapetal and nontapetal retina and vacuoles formed between cells [88]. At 6 to 9 years of age, dense inclusions were found in the retinal pigment epithelium, and peripheral photoreceptor degeneration was also recognized. Storage body accumulations were also recognized in the brain around 3 years of age. There was a loss of cerebellar Purkinje cells and brain stem neurons contained large numbers of cytoplasmic storage bodies. The cerebellar cortex had the heaviest concentration of storage bodies. All inclusions had granular and lamellar forms. Tibetan Terrier NCL appeared to be an autosomal recessive trait. The gene mutation responsible for this disease is not known [66, 88, 89].

Welsh Corgis

An adult onset NCL occurs in Welsh Corgis. Usually, symptoms in adult onset NCLs progress gradually. However, the age of onset of Welsh Corgis was 6

to 8 years of age, and the course of the disease showed rapid terminal deterioration. The dog showed a loss of coordination, hindlimb weakness, a progressive loss of proprioception, hypersensitivity to sound, hyperexcitability, jaw champing, head pressing, and seizures. Brain atrophy was recognized but any other pathological features are unknown [90].

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Chapter III: A mutation in the *CLN8* gene in English Setter dogs with neuronal ceroid-lipofuscinosis

Abstract

A heritable neurodegenerative disease of English Setters has long been studied as a model of human neuronal ceroid-lipofuscinosis (NCL). Megablast searches of the first build of the canine genome for potential causative genes located the *CLN8* gene near the q telomere of canine chromosome 37, close to a marker previously linked to English Setter NCL. Sequence analysis of the coding region from affected dogs revealed a T-to-C transition in the *CLN8* gene that predicts a p.L164P missense mutation. Leucine 164 is conserved in four other mammalian species. The C allele co-segregated with the disease phenotype in a two-generation English Setter family in a pattern consistent with autosomal recessive inheritance. All four NCL-affected family members were C/C homozygotes and all four obligate carriers were C/T heterozygotes; whereas, 103 unrelated dogs were all T/T homozygotes. These findings indicate that the *CLN8* T-to-C transition is the likely cause of English Setter NCL.

Introduction

The neuronal ceroid-lipofuscinoses (NCLs) are a group of autosomal recessively inherited lysosomal storage disorders that occur in people, and in laboratory and domestic animals, including dogs [1], [2] and [3]. The NCLs are characterized by substantial accumulation of autofluorescent lysosomal storage bodies in the cells of many tissues and by progressive neurodegeneration that results in severe neurological impairment and premature death. Different forms of human NCL result from mutations in one of at least six different genes: palmitoyl-protein thioesterase 1 (*PPT1*), *CLN2*, *CLN3*, *CLN5*, *CLN6*, and *CLN8* [1] and [4]. In addition, a naturally occurring NCL of sheep has been associated with a mutation in the cathepsin D gene (*CTSD*) [5], and transgenic mice with a homozygous knockout of their chloride channel 3 gene (*CLCN3*) develop an NCL-like disease [6].

Efforts to develop therapeutic interventions for the human NCLs will be greatly enhanced by the availability of appropriate animal models. Mouse models for most of the NCLs have been developed since the characterization of the human NCL genes [7], [8], [9], [10], [11] and [12]. However, the utility of

these mouse models is limited because, in some cases, affected mice do not develop phenotypic features of the diseases seen in the corresponding human disorders [13]. Furthermore, the small size, primitive nervous system, and limited cognitive capacity of mice limit their usefulness in studies of mechanisms of disease pathology and in therapeutic intervention studies utilizing gene replacement strategies [14] and [15]. Therefore, it would be very useful to have other animal models for the NCLs.

In the 1950s, the Norwegian veterinarian Nils Koppang discovered an NCL-like disease in a group of related English Setters [16]. He established a breeding colony of these dogs, which is still being maintained [17]. Although the phenotypic features of the English Setter disease have been extensively characterized [16], [18], [19], [20], [21] and [22], the genetic basis for NCL in these dogs has not previously been reported. Mutations in the canine orthologs of both the *CLN2* and *CLN3* have been excluded as the cause of NCL in the English Setters [23] and [24]. Lingaas et al. [25] mapped the English Setter NCL locus to canine chromosome 37 (CFA37). Maps that compared the canine and human genomes indicated that CFA37 is completely orthologous to a region of human

chromosome 2 (HSA2) [26], [27], [28] and [29]. Since none of the eight above-mentioned candidate genes map to HSA2, it appeared that the NCL of English Setters was caused by a mutation in an as yet undiscovered NCL gene. Here we report the existence of a previously unrecognized segment of sequence conservation between a region of HSA8 and a region of CFA37. We further report that *CLN8*, which lies within this conserved chromosomal segment, contains a missense mutation in English Setters with NCL.

Materials and methods

Canine DNA was prepared from white blood cell pellets, obtained from EDTA-blood after lysis of the red blood cells with 0.15 M NH_4Cl . The white blood cells were lysed with 0.5% SDS and protein was precipitated with 5.7 M NH_4Ac . DNA was precipitated from the supernatant with 50% isopropanol, washed with 75% ethanol, resuspended in Tris/EDTA buffer, and stored frozen until use. Eight of the DNA samples were from related English Setters in the University of Indiana's canine NCL colony. Other DNA samples were selected

from unrelated dogs represented in the University of Missouri Canine DNA Repository. Among the selected DNAs were samples from an NCL-affected Tibetan Terrier and an NCL-affected American Bulldog. The diagnosis of canine NCL was based on behavioral signs of neuronal and retinal degeneration and the demonstration of autofluorescent intracellular granules in brain tissue sections [30] and [31].

PCR primer pairs 5'-CAGACGGATACATGAAGGTCCGAGGGG-3' with 5'-AGCACGGGGTCCACCAGCAAGG-3' and 5'-CTGGAACCTGGC GGCCACG-3' with 5'-CCCCACGCCCTGTGAGAAACACT-3' were used to produce overlapping amplicons which included the coding sequences from canine *CLN8* exon 2. A gene segment which included the coding sequences from canine *CLN8* exon 3 was amplified with PCR primer pair 5'-AAAGTGAAGAG TGTTGATGGTCGTGCGTT-3' with 5'-AAATTCACATGAAGGACACGGTCC GACT-3'. The amplicons were sequenced in both directions as previously described [32]. Pyrosequencing was performed with a PSQ 96 Pyrosequencer as directed in the PSQ Sample Preparation Guidelines for SNP Analysis Manual (Biotage). The PCR primers were 5'-biotinyl-CGGCCACTATCTGGCTATG-3'

with 5'-CAGGAAATGCAGGTGAAGG-3'; and, 5'-TCGCCTCCAGAAGC-3' was used for the sequencing primer.

Chromosomal locations of canine orthologs of NCL candidate genes were determined by using candidate-gene coding sequences in a megablast query of the first build of the canine genome at

http://www.sciencedirect.com/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_cdi=6713&_plusSign=%2B&_targetURL=http%253A%252F%252Fwww.ncbi.nlm.nih.gov%252Fgenome%252Fseq%252FCfaBlast.html. The

position of the canine microsatellite marker AHT135 in the canine genome was determined by a blastn query of the first build of the canine genome with AHT135 primer sequences

(http://www.sciencedirect.com/science?_ob=RedirectURL&_method=externObjLink&_locator=url&_cdi=6713&_plusSign=%2B&_targetURL=http%253A%252F%252Fwww.ncbi.nlm.nih.gov%252Fgenome%252Fsts%252Fsts.cgi%253Fuid%253D262020).

The relative positions of features in CFA37 and in CFA27, and the distances between them were estimated from their respective chromosomal assemblies (GenBank Accession Nos. CM000037 and CM000027). Individual

contigs from these canine chromosomes were used in megablast queries

http://www.sciencedirect.com/science?_ob=RedirectURL&_method=externObjLink&locator=url&_cdi=6713&_plusSign=%2B&_targetURL=http%253A%252F%252Fwww.ncbi.nlm.nih.gov%252Fgenome%252Fseq%252FHsBlast.html) to

identify corresponding human genome locations.

Results

Megablast queries of the first assembly of the canine genome with human candidate gene coding sequences revealed the locations of orthologous genes in the canine chromosomes (Table 1). The unexpected finding that canine *CLN8* was located in contig AAEX01055130 near the q-arm telomere of CFA37 was confirmed by using adjacent CFA37 contigs in megablast queries of the human genome. This analysis revealed that sequences orthologous to the peri-telomeric region of HSA8p are contained in a CFA37 chromosomal segment of approximately one megabase that extends telomeric from contig AAEX01055114. Canine microsatellite marker AHT135, the marker most closely linked to the

English Setter NCL locus [25], was found in canine contig AEX01055070. This contig contains HSA2 orthologs but is located only about three megabases centromeric from the canine *CLN8* gene in CFA37.

Canine *CLN8* segments from exons 2 and 3 that include the entire *CLN8* coding sequence were amplified from DNA from two NCL-affected English Setters and sequenced. The coding sequences from both samples were identical to those in contig AAEX01055130 from the Boxer sequenced in the Canine Genome Project, except that each contained a c.491C>T transition which predicts a p.L164P missense mutation (Fig. 1). A pyrosequencing assay that distinguished T/T, C/T, and C/C genotypes (Fig. 2) was used to analyze DNA samples from eight English Setters from the University of Indiana canine NCL colony. All four clinically affected dogs in this family were C/C homozygotes and all four obligate carriers (clinically normal parents or offspring of a dog with recessive NCL) were C/T heterozygotes (Fig. 3). The pyrosequencing assay was also used to genotype 3 unrelated normal English Setters from the pet population and 100 dogs representing 100 other breeds. Each of these 103 dogs proved to be a T/T homozygote, indicating that the C allele is rare in the canine population.

The coding regions of *CLN8* exons 2 and 3 were also amplified from DNA from an NCL-affected Tibetan Terrier and an NCL-affected American Bulldog. The *CLN8* coding sequences from both of these dogs were identical to sequences from the above-mentioned Boxer.

Because the detected orthology between the peri-telomeric region of canine CFA37 and HSA2 (Fig. 4A) had not been previously reported, megablast searches were performed to determine if the peri-telomeric regions of other canine chromosomes contained additional unreported segments of conserved human/canine synteny. Human orthologs of the peri-telomeric regions of all canine chromosomes except CFA27 and CFA37 were found to be consistent with current human/canine comparative maps [26], [27], [28] and [29]. Although most of CFA27 is orthologous to a region of HSA12, orthologs of human genes from the peri-centromeric region of HSA22q were found in CFA27 starting at contig AAEX01027081 and continuing in adjacent telomeric contigs (Fig. 4B).

Discussion

Although there are substantial overlaps in neurological signs among most human patients with NCL, there are sufficient differences, primarily in the age of clinical onset and rate of disease progression, that the NCLs were classified as infantile, late-infantile, and juvenile forms long before the underlying gene defects were identified [33] and [34]. Extensive phenotypic characterization of the English Setter NCL led to the conclusion that the canine disorder most closely resembled the human juvenile disease [16]. In 1995, the gene that is defective in most human subjects with the juvenile form of NCL was identified and designated *CLN3* [35]. Since 1995, mutations in *PPT1*, *CLN2*, *CLN5*, *CLN6*, and *CLN8* have also been associated with various forms of human NCL [1], [12], [36], [37], [38] and [39]. These, along with *CTSD* and *CLCN3* which have caused NCL-like diseases in sheep and transgenic mice [5] and [6], have been considered candidates for the NCL gene in English Setters. Both *CLN2* and *CLN3* have previously been excluded as English Setter NCL loci by sequence and linkage analyses [23] and [24]; furthermore, biochemical analyses indicate that English Setter NCL is unlikely to be the result of a mutation in canine *PPT1* [20] and [40].

The report by Lingaas et al. [25] that English Setter NCL was genetically linked to microsatellite marker AHT135 on CFA37 with a recombination fraction of 0.029 and the placement of *CLN8* in the first build of the canine genome at a location about 3 megabases telomeric to AHT135 made *CLN8* a likely candidate to be the English Setter NCL locus. Comparison of the entire *CLN8* coding sequences from NCL-affected English Setters with that from the Boxer sequenced in the Canine Genome Project revealed a c.491T>C transition. This transition predicts a p.L164P missense mutation. The C allele co-segregated with the disease phenotype in a two-generation English Setter family in a pattern consistent with autosomal recessive inheritance. One hundred and three unrelated dogs including three English Setters and 100 dogs representing 100 different breeds were all homozygous for the T allele. The leucine at position 164 is conserved in other mammalian species (Fig. 5). Taken together, these observations strongly suggest the T-to-C transition at position 491 in the canine *CLN8* is the cause of English Setter NCL. On the other hand, no *CLN8* missense mutations were detected in DNA from either an NCL-affected American Bulldog

or an NCL-affected Tibetan Terrier, suggesting that mutations at loci other than *CLN8* are responsible for the NCL in these breeds.

Human *CLN8* encodes a polypeptide of 286 amino acids that was predicted to be a transmembrane protein [12]. It has been reported that in some cell types this protein (CLN8) is resident in the endoplasmic reticulum (ER) and recycles between the ER and ER-Golgi intermediate compartment, whereas in other cells CLN8 is associated with a membrane fraction different from the ER [42] and [43]. CLN8 belongs to the family of membrane-associated proteins that contain the TRAM-Lag1p-CLN8 or TLC domain [41]. Some other TLC-domain containing homologs are involved in regulating ceramide synthesis or translocating proteins across ER membranes [41]; however, the function of CLN8 has not been established. The Winter and Ponting [41] structural model for CLN8 places leucine 164 within the protein's fourth transmembrane helix. If this model is correct, it is likely that mutant proline 164 alters CLN8 function by disrupting the secondary structure of the fourth transmembrane helix.

The known NCL-associated mutations in human *CLN8* result in the following predicted amino acid changes: R24G; W263C; R204C; [L16M; T170M];

and, a frame shift and truncation starting at codon 30 due to a c.88delG single nucleotide deletion [12] and [36]. The disease that results from the p.R24G amino acid substitution is known as Northern epilepsy and has been reported only in Finland [12]. The clinical phenotype in Northern epilepsy is distinct from other forms of NCL. Symptoms first present between 5 and 10 years of age as frequent tonic-clonic seizures followed by progressive mental retardation. Clinical progression is slow, and there is little if any vision loss [12] and [36]. In contrast to Northern epilepsy, all of the other *CLN8*-associated diseases reported to date are from Turkish patients who show a more rapid progression with typical NCL symptoms including seizures, motor impairment, myoclonus, mental regression, and loss of vision [36]. Thus, the Turkish patients with *CLN8* mutations cannot be distinguished clinically from many NCL patients with diseases resulting from mutations in genes other than *CLN8*. Because the identities of genes responsible for many cases of human NCL worldwide are yet to be determined, it is likely that some patients from geographic locations outside of Turkey and Finland have NCL resulting from mutations in *CLN8*.

Affected English Setters appear normal at birth; however, between 1 and 2 years of age they begin to exhibit NCL-like symptoms. These include seizures, progressive motor and cognitive decline, and visual impairment [16] and [18]. Affected dogs typically die at approximately 2 years of age due to intractable seizures. These symptoms mimic fairly closely the clinical phenotype reported for Turkish patients with *CLN8* mutations [36]. NCL has also been reported in the *mnd* mouse, which has a frameshift mutation in the murine ortholog of *CLN8* [12]. Affected mice exhibit loss of vision due to retinal degeneration and also suffer from a progressive decline in motor functions [44] and [45]; however, spontaneous seizures have not been reported in *mnd* mice nor has cognitive impairment been identified in this model. Thus, at least with respect to clinical signs, the English Setter appears to be a better model for human *CLN8*-associated NCL than does the *mnd* mouse. It should be noted that the lysosomal storage bodies that accumulate in people, mice, and dogs with *CLN8* mutations all contain large amounts of mitochondrial ATP synthase subunit c [20], [37] and [46], suggesting that similar biochemical pathways are disrupted in all three species.

The English Setters may prove to be useful not only for studying NCL disease mechanisms, but also for developing therapeutic interventions. Therapeutic interventions that are developed using the English Setter model may prove applicable not only to human subjects with *CLN8* mutations, but also to the treatment of other forms of NCL that result from mutations in genes that encode intrinsic membrane proteins of unknown function (*CLN3*, *CLN5*, and *CLN6*).

Breen et al. [27] described a total of 79 chromosomal segments with conserved synteny between the human and canine genomes. Our analyses have resulted in identification of two additional regions of conserved synteny: between HSA8 and CFA37, and between HSA22 and CFA27. A detailed understanding of the comparative organizations of the canine and human genomes will aid in identifying the loci controlling other traits in both species.

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Table 1.

Comparative genome map locations of the human and canine orthologs of known and putative NCL genes

Candidate gene	Human chromosome	Canine contig^a	Canine chromosome
<i>PPT1</i>	HSA1p	AAEX01015067	CFA15
<i>CLN2</i>	HSA11p	AAEX01017594	CFA21
<i>CLN3</i>	HSA16p	AAEX01044199	CFA6
<i>CLN5</i>	HSA13q	AAEX01010623	CFA22
<i>CLN6</i>	HSA15q	AAEX01008667	CFA30
<i>CLN8</i>	HSA8p	AAEX01055130	CFA37
<i>CTSD</i>	HSA11p	AAEX01053128	CFA18
<i>CLCN3</i>	HSA4q	AAEX01020012	CFA25

^a GenBank accession numbers.

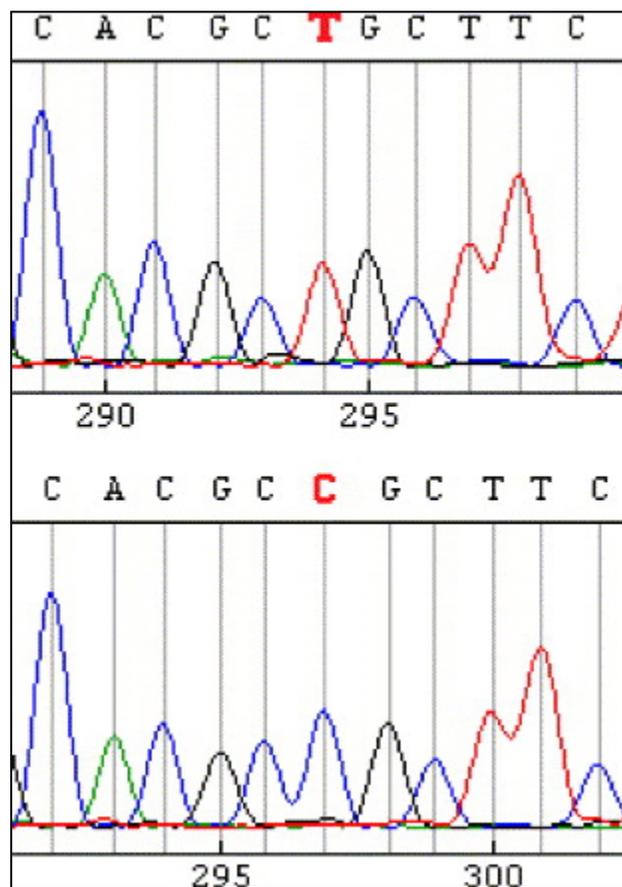


Fig. 1. Aligned sequence chromatograms showing *CLN8* segments from a dog that is homozygous for the wild-type or T allele (top) and from an affected English Setter, homozygous for the mutant or C allele (bottom). Genomic sequences that include the entire *CLN8* coding sequence from exons 2 and 3 from an NCL-affected English Setter are available in GenBank: Accessions Nos. AY785293 and AY785294, respectively.

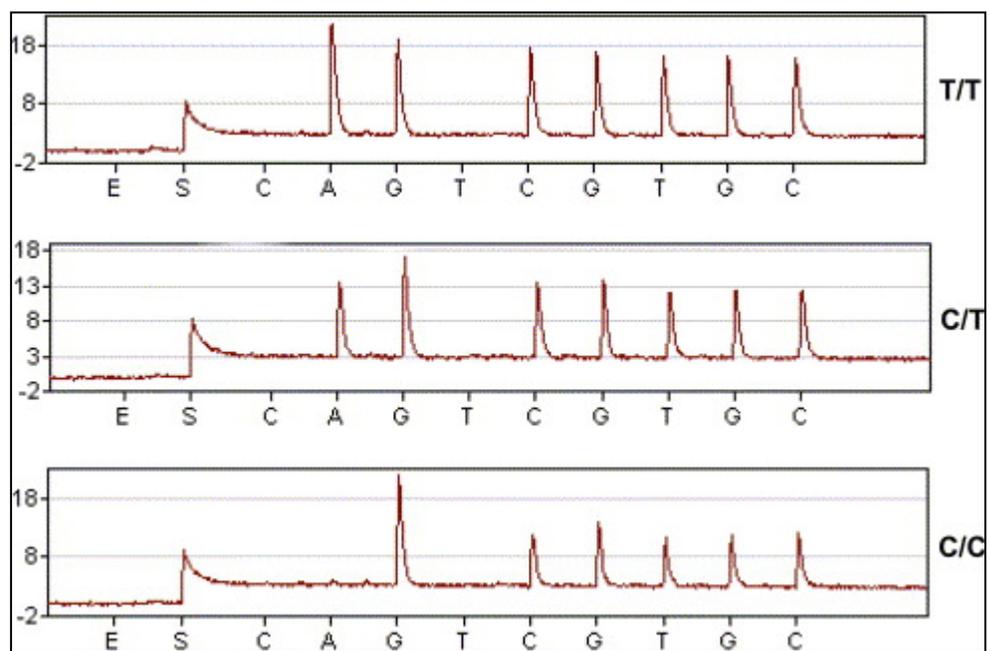


Fig. 2. Pyrosequograms produced in the antisense direction: from a T/T homozygote (top), a C/T heterozygote (center), and a C/C homozygote (bottom). See Ronaghi [47] for interpretation.

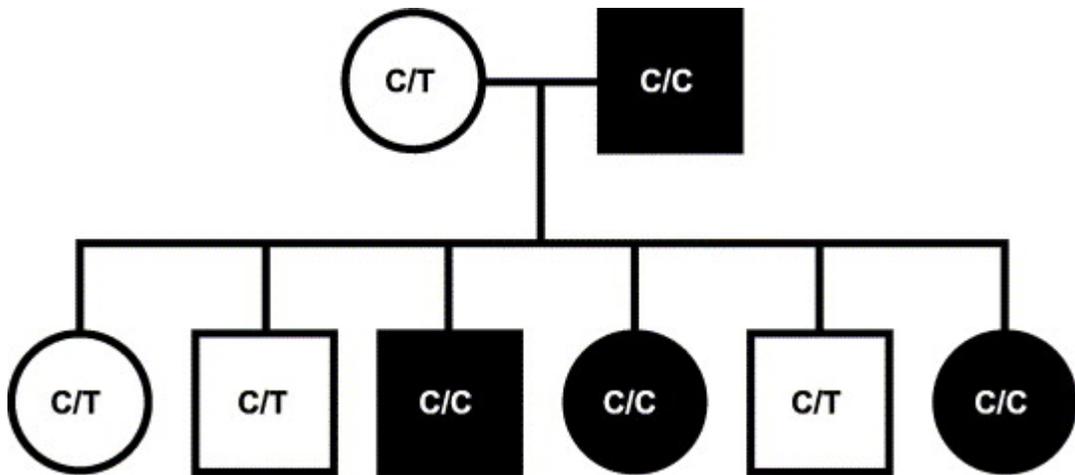


Fig. 3. A pedigree from a family of English Setters showing the patterns of inheritance of the NCL phenotype and the two *CLN8* alleles. Solid figures represent dogs with clinical NCL; open figures represent clinically normal dogs.

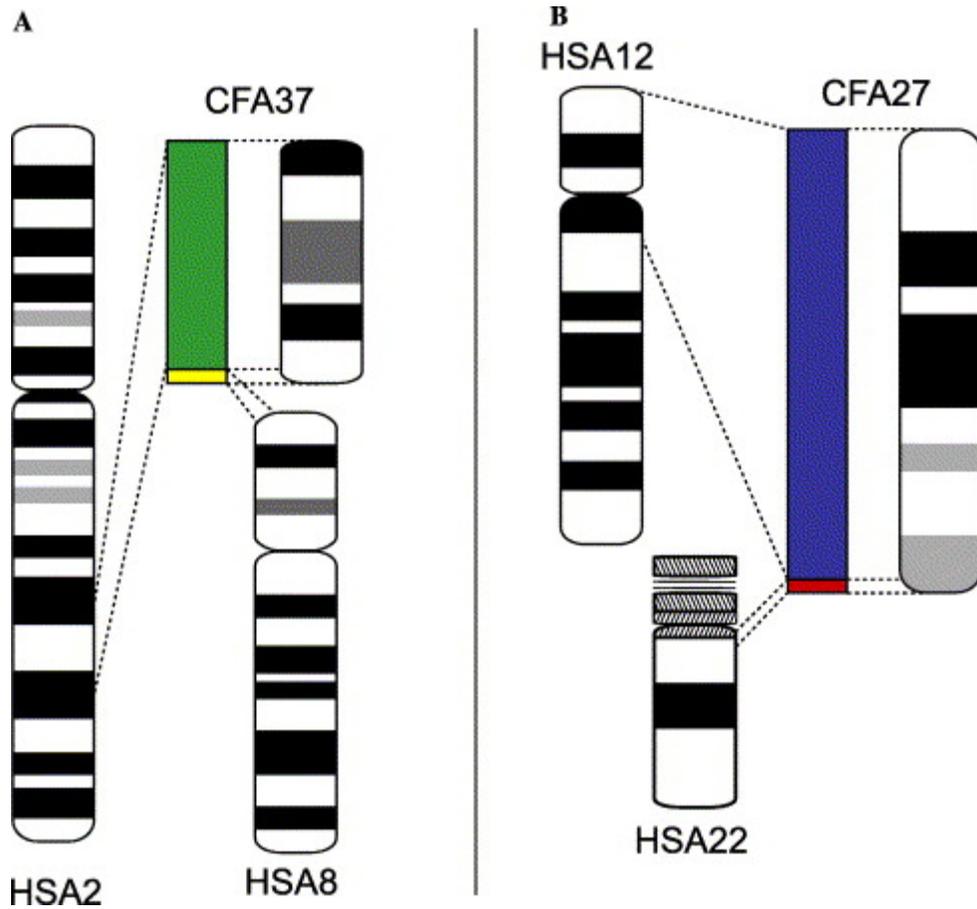


Fig. 4. Idiograms showing the chromosomal locations of regions of sequence conservation between (A) CFA37 and segments of HSA2 and HSA8; and, (B) CFA27 and segments of HSA12 and HSA22.

Human	156	GHYLAMTTLLLEMSTPFTCVSWMLLK	181
Mouse	156	GHYLAMTTLLLEMSTPFTCISWMLLK	181
Rat	156	GHYLAMTTLLLEMSTPFTCVSWMLLK	181
Cow		GHYLEPMVTLLEEMSTPFACISWMLLK	
Dog	156	GHYLAMSTLLEASTPFTCISWMLLK	181
NCL/ES	156	GHYLAMSTELLEASTPFTCISWMLLK	181

Fig. 5. Aligned amino acid sequences for segments of *CLN8* from five mammalian species. The human, mouse, and rat sequences are from GenBank Accession Nos. NM_018941.2, NM_012000.2, and XM_225033, respectively. The cow sequence was predicted from the assembly of TI Nos. 423801437, 381231032, and 515261501 from the NCBI *Bos taurus*-WGS trace archives. The dog sequence was predicted from CFA37 contig AAEX01055130.1 and the sequence from the NCL-affected English Setter (NCL/ES) containing Pro164 (black background) was predicted from sequences determined in the present study. The gray highlighting identifies residues that differ from the consensus sequence.

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Chapter IV: A mutation in the cathepsin D gene (CTSD) in American Bulldogs with neuronal ceroid-lipofuscinosis

Abstract

We obtained DNA, brains, and eyes from American Bulldogs with neurodegeneration due to neuronal ceroid lipofuscinosis (NCL). The diagnosis of NCL was confirmed by detection of autofluorescent cytoplasmic inclusions within neurons throughout the brains, in retinal ganglion cells, and along outer limiting membranes of the retinas. Electron microscopy revealed that the inclusions had coarsely granular matrices surrounding well-delineated spherical structures and that the inclusions near the retinal outer limiting membranes were within photoreceptor cells, mostly cones. Affected American Bulldogs were homozygous for the A allele of a G to A transition in the cathepsin D gene (CTSD), which predicts the conversion of methionine-199 to an isoleucine. Only the G allele was detected in DNA samples from 131 randomly selected dogs representing 108 breeds other than American Bulldog; however, the A allele had a frequency of 0.28 among 123 genotyped American Bulldogs. Transmission analysis in a 99 dog pedigree of American Bulldogs indicated a probability of less than 10^{-7} that alleles from any mutation unlinked to CTSD would be concordant

with the pedigree and phenotypes of the dogs. Brain samples from affected dogs had 36% of the cathepsin D-specific enzymatic activity found in control dog brains; whereas, specific enzymatic activities of 15 other lysosomal enzymes were unchanged or increased. Compared to previously described NCLs in mice and sheep that completely lack cathepsin D activity, the clinical course of NCL in the American Bulldogs was less severe and more closely resembled that of many human NCLs.

Introduction

The neuronal ceroid lipofuscinoses (NCLs) are a group of heritable diseases characterized by progressive neuronal degeneration and the accumulation of autofluorescent cytoplasmic inclusions in the brain, retina, and other tissues. NCLs have been described in people, mice, and a variety of domestic mammals including sheep and dogs. Human NCLs have been attributed to mutations in six genes: PPT1, CLN2, CLN3, CLN5, CLN6, and CLN8 [1-7]. Mutations in three additional genes are associated with NCL in animals. Sheep homozygous for a missense mutation in the cathepsin D gene

(CTSD) are born with severe NCL [8]. Furthermore, knockout mice rendered nullizygous for CTSD, CLCN3 or PPT2 develop forms of NCL [9-11]. Canine NCLs have been diagnosed in at least 18 breeds where they are important both as veterinary diseases and as potential models for human NCLs (http://www.caninegeneticdiseases.net/CL_site/formsCL.htm). Previous studies have found a CLN8 missense mutation associated with NCL in English Setters [12] and a CLN5 nonsense mutation associated with NCL in Border Collies [13]. A young-adult onset NCL occurs in American Bulldogs [14]. Affected dogs typically exhibit hypermetria and ataxia before two years of age. Psychomotor deterioration progresses slowly but relentlessly, leading to death before seven years of age. Cytoplasmic autofluorescent storage material is present within neurons in the brains and retinal ganglion cells of affected dogs [14]. We obtained DNA from affected individuals and began sequencing exons from the candidate genes mentioned above. We here report evidence that a missense mutation in CTSD is responsible for NCL in American Bulldogs.

Materials and methods

One hundred and twenty-three EDTA-anticoagulated blood samples were obtained from privately owned American Bulldogs and DNA was extracted as previously described [12]. The owners of these dogs provided pedigree information and submitted a form detailing their animal's relevant clinical history (<http://www.caninegeneticdiseases.net/CLsite/formsCL.htm>). Three of the American Bulldogs were available for necropsy as described below. Another 131 canine DNA control samples representing 108 breeds other than American Bulldog were randomly selected from the University of Missouri Canine DNA Repository. To confirm the clinical diagnoses and further characterize American Bulldog NCL, the brains and eyes of three terminally affected American Bulldogs were collected at necropsy, immediately after the dogs were euthanized. Brain samples from these dogs were promptly frozen and stored at - 70 °C as were similarly collected brain samples from four age-matched normal control dogs. The eyes and portions of the cerebral cortex and cerebellum were preserved for fluorescence microscopy (FM), and electron microscopy (EM). Cryostat sections obtained from the FM samples were examined for storage body autofluorescence

as previously described [15]. Ultrathin tissue sections were examined and photographed with a JEOL 1200 EX transmission electron microscope. All 9 CTSD exons were amplified by PCR with primers designed from sequences in GenBank contig numbers AAEX01015066.1 and AAEX01015067.1 which contain the gene. Annealing temperatures and oligonucleotide sequences for each primer pair are listed in Table 1. Because of the high G/C content in and around exon 1, 1M betaine was included in the amplification reaction mixture for this exon. Amplicons were sequenced in both directions as previously described [16]. Pyrosequencing with a PSQ 96 instrument was used for genotyping the DNA samples with respect to a missense mutation in the CTSD gene. The PCR primers were 5'-biotinyl TTCATCGCGGCCAAGTT-3' with 5'-TGTCGAAGACAGGCA GCAC-3'; and, the sequencing primer was 5'-GATGCGGGGGTAGGC-3'. Enzyme-specific activities in cerebral cortex tissues from three American Bulldogs with terminal NCL and from four age-matched control dogs were determined as previously described [8]. We constructed an extended pedigree of 56 genotyped dogs aged 2 years and greater for which phenotype was known without error (8 affected and 48 normal) and 43 non-genotyped relatives, which

provided pedigree relationships among the genotyped animals. We then derived the probability that a randomly sampled biallelic mutation would be concordant with the pedigree and phenotypes as a function of the unknown minor allele frequency q . For this calculation, all obligate carriers were uniquely assigned heterozygous genotypes, all affected animals were assigned homozygous genotypes for the minor allele (assumed to be the disease-associated allele), and all other animals with normal phenotypes were allowed to be either heterozygous or homozygous for the major allele. Genotype probabilities were computed for each animal within the pedigree based upon transmission probabilities and the assumption that the minor allele was the recessive disease-causing mutation. The probability that a randomly sampled biallelic mutation was concordant with the pedigree and phenotypes as:

$$PC = \sum_S \prod_i P_S(P_i | G_i) P_S(G_i),$$

where $P_S(G_i)$ is the probability of the genotype of the i th individual within the pedigree, $P_S(P_i | G_i)$ is the probability of the phenotype of the i th individual given the individual's genotype and the summation is across all possible sets (S) of genotypes that are concordant with the pedigree and phenotypes. Since, we assume that the genotypes are fully penetrant, $P_S(P_i | G_i)$

=1 for all genotypes that are concordant with the observed phenotype. Consequently, P_C reduces to $\sum_s \prod_i P_s(G_i)$. This probability was maximized ($P_C=10^{-13.64}$) at $q=0.27$ which is consistent with the value of 0.28 obtained from the total sample of 123 genotyped individuals. From this, we estimate the probability that there is no other mutation within the dog genome that is concordant with the pedigree and phenotypes as $P_0 = (1 - P_C)^N$, where N is the number of independent biallelic mutations within the dog genome. Thus, the probability that there are any other mutations within the dog genome that are consistent with the pedigree and phenotypes is $1 - P_0$.

Results

All three of the affected American Bulldogs available for necropsy had massive accumulations of autofluorescent storage bodies in the cerebellum, cerebral cortex, and retina. In addition to the substantial accumulations of storage material in the retinal ganglion cells, autofluorescent inclusions were observed along the outer limiting membrane of the retina adjacent to the outermost layer of photoreceptor nuclei (Fig. 1). At the ultrastructural level, the

storage bodies were observed to be membrane-bound cellular inclusions with cross-sectional diameters generally ranging from 0.5 to 3 μ m (Fig. 2). The inclusions had coarsely granular matrices. Often embedded within the matrices were well-delineated spherical inclusions that were either more darkly or more lightly stained than the surrounding matrix. With EM it could be seen that the storage bodies near the outer limiting membrane were in photoreceptor cells, with the majority of the inclusions occurring within cone cells (Fig. 2B). The ultrastructural appearances of the storage bodies in the photoreceptor cells were similar to those previously reported in other neural cell types in the affected American Bulldogs [14]. Nucleotide sequences were determined for all nine CTSD exons from an American Bulldog confirmed with FM and EM analyses to have NCL. These sequences were compared to corresponding sequences in GenBank contigs AAEX01015066.1 and AAEX01015067.1 from the Boxer DNA sequence produced in the canine genome project (<http://www.genome.gov/12511476>). The American Bulldog sequences from exons 1, 2, 3, 4, 6, 7, 8, and 9 were identical to the Boxer sequences; however, the exon 5 sequence contained a transition, c.597G>A, which predicts a p.M199I

missense mutation (Fig. 3A). Using a pyrosequencing assay that distinguishes the A and G alleles (Fig. 3B), we found that all three of the NCL-affected American Bulldog samples available to us at the initiation of this project were homozygous for the A allele; whereas, the A allele was completely absent in 131 randomly selected dogs representing 108 other breeds. We subsequently obtained and genotyped DNA from 120 additional American Bulldogs including five that exhibited clinical signs of NCL. The frequency of the A allele was 0.28 among the 123 American Bulldogs genotyped in this study. In this group of American Bulldogs, the clinical histories of 66 G/G homozygotes and 45 G/A heterozygotes were unremarkable. Eight of the 12 A/A homozygotes exhibited neurological signs of NCL. The other four A/A homozygotes were less than four months old and had not yet shown signs of the disease. The amino acid sequence for canine cathepsin D was translated from the canine CTSD exon sequences and used to query the non-redundant coding sequence translations in GenBank in a BlastP search for cathepsin D orthologs. As shown in Table 2, the methionine at amino acid position 199 of the canine protein was conserved in the cathepsin D sequences of 18 of 19 vertebrates and in all eight mammalian species identified

by the query. The one exception among the vertebrates was the Italian wall lizard, *Podarcis sicula*, in which the methionine was replaced by a leucine. The BlastP search also identified 13 cathepsin D orthologs from a diverse group of invertebrates, 11 of which had a methionine residue at the corresponding position in the protein. The two exceptions were sequences from mosquitoes, which like the Italian wall lizard, had leucine substituted for the methionine. The mean cathepsin D-specific activity from three brains from NCL-affected American Bulldogs was reduced to 36% of the mean activity from four brains from control dogs ($P=0.001$); whereas, the affected dog activities of 15 other lysosomal enzymes were either unchanged ($N=9$) or exhibited a significant increase ($N=6$; Table 3). Fig. 4 shows the relationships and genotypes of a 99 member American Bulldog family segregating NCL. We assume that the number of biallelic mutations within the American Bulldog genome is $N=3 \times 10^6$. This estimate is consistent with the 2.1 million single nucleotide polymorphisms detected in the first assembly of the whole genome sequence for dog (<http://www.broad.mit.edu/mammals/dog/>) and leads to an estimate of $P_0=0.99999993156$ if all mutations occur with a minor allele frequency of $q=0.27$

and $P_0=0.99999997657$ if mutations are assumed to have minor allele frequencies uniformly distributed between (0,0.5]. Consequently, the probability that there is at least one other mutation within the dog genome that is consistent with the pedigree and phenotypes ($1- P_0$) is less than 10^7 .

Discussion

Previously described mutations in CTSD cause early onset NCL in two animal models [8,9]. CTSD knockout mice have been studied since 1995 [17]. Nullizygotes appear normal at birth and grow normally for about two weeks, but develop lymphopenia, thromboembolia, and intestinal necrosis leading to death at 25–27 days of age [17]. Koike et al. [9] noted that in the terminal stages of their disease, these mice had seizures and loss of vision and found that neurons in the brains of these mice accumulated cytoplasmic autofluorescent storage granules consistent with NCL. Tyynela et al. [8] studied sheep with a naturally occurring congenital NCL and identified a missense mutation in CTSD, that converted an active site aspartic acid codon to an asparagine codon. The mutant CTSD produced an inactive but stable cathepsin D protein. New born

homozygous affected lambs were weak and unable to stand, but some could suckle and survive for a few weeks with bottle feeding [18]. At birth their brains were grossly underdeveloped and about half normal size [8]. Neurons from these brains contained cytoplasmic autofluorescent storage granules [8,18]. The cells of visceral organs also accumulated storage granules; however, affected sheep did not develop the lymphopenia, thromboembolia, and intestinal necrosis observed in the CTSD knockout mice. Brain tissues from homozygous affected sheep lacked detectable cathepsin D activity; whereas, the activities of 12 other lysosomal enzymes were normal or increased in these tissues [8]. Most of the clinical, histological, and ultrastructural characteristics of American Bulldog NCL were described in an earlier report [14]. As in that previous study, we found that autofluorescent lysosomal storage material accumulates in neurons throughout the brains of affected American Bulldogs and in their retinal ganglion cells. In addition, we detected autofluorescent inclusions near the outer limiting membrane of the retina due to the accumulation of storage material in the photoreceptor cells, especially the cones. To our knowledge, there are no previous reports of cytoplasmic autofluorescent inclusions within photoreceptor

cells in conjunction with any of the human or animal NCLs. If this photoreceptor pathology is, in fact, unique to NCL caused by CTSD mutations, it would imply that cathepsin D is essential for normal protein turn over in photoreceptors; whereas, there is no comparable requirement for those gene products that are abnormal or missing in the other NCLs. It is not yet known whether the accumulation of storage material in the photoreceptors is associated with impaired photoreceptor cell function. The ultrastructural appearance of the cytoplasmic inclusions in most previously described human and animal NCLs have been classified as curvilinear, fingerprint, or rectilinear profiles or as granular osmophilic deposits [19]. Unlike these classical ultrastructures, the storage bodies in the American Bulldog disease observed here and in an earlier study [14] consisted of round uniformly staining inclusions embedded within granular matrixes. They resemble the inclusion body ultrastructures from the CTSD knockout mice [8] and certain atypical human NCLs [20]. An adult-onset neurodegenerative disease with some features of NCL was recently reported in two breeds that are closely related to the American Bulldog: the American Staffordshire Terrier and the Pit Bull Terrier [21]. In the latter breeds,

neurodegeneration is accompanied by the accumulation of cytoplasmic autofluorescent storage material; however, the storage material accumulation and neurodegeneration were found only in cerebellar Purkinje cells and in certain thalamic nuclei. This restricted distribution of lesions differs greatly from the wide-spread distribution, which is observed in brains from affected American Bulldogs [14] and which is characteristic of all previously described NCLs. Furthermore, curvilinear and fingerprint profiles are prominent ultrastructural features of the storage material in the American Staffordshire/Pit Bull Terrier disease [21]; whereas, the American Bulldog storage material has the atypical granular ultrastructure described above. Thus, the American Bulldog NCL is distinct from the neurodegenerative disease described in American Staffordshire and Pit Bull Terriers. We have identified a G or A transition within the canine CTSD gene. Although, the A allele appears to be rare or absent in other breeds, it was found in 28% of the chromosomes from the 123 American Bulldogs genotyped in this study. Eight of these American Bulldogs exhibited clinical signs of NCL and all eight were A/A homozygotes. The four other A/A homozygous American Bulldogs were all less than four months of age and

presumed to be preclinical. Since, DNA samples were not available from many of the siblings and ancestors of the affected dogs, we were unable to perform a linkage analysis to demonstrate a twopoint association between the disease locus and the c.597G>A mutation. Instead, we performed a transmission analysis in which we estimated the probability that a randomly sampled biallelic mutation would be concordant with the pedigree and known phenotypes of dogs aged two years old or older. From this probability, we estimated the probability that there would be at least one mutation unlinked to CTSD in the genome concordant with the pedigree and phenotypes and demonstrated that this event was extremely unlikely. We therefore conclude that, there is strong evidence supporting the identity of c.597G>A, or a tightly linked mutation, as the cause of NCL in American Bulldogs. The c.597G>A transition is a missense mutation in exon five of canine CTSD that converts methionine-199 to an isoleucine. Methionine-199 in the canine cathepsin D amino acid sequence is preceded by an eight amino acid sequence, AKFDGILG, that is conserved in cathepsin D sequences from all of the other 31 diverse species identified by a BlastP search of the non-redundant translated Gen-Bank sequences (Table 2). This eight amino

acid motif is followed by methionine in 29 of the 32 cathepsin D sequences. The three exceptions are from a lizard and two species of mosquito; and, in all three exceptions the amino acid that replaces the methionine is leucine. Canine cathepsin D is an aspartate protease and aspartic acids at positions 97 and 293 of the canine protein contribute to the active site [22]. The functional role of methionine-199 and nearby amino acids is unknown; however the conservation of the adjacent amino acid sequence across every species for which sequence was detected implies that the region is under strong purifying selection and thus, has a functional role. The substitution of isoleucine for methionine at position 199 is a conservative change; however, the association of this mutation with NCL suggests that this substitution may affect the biosynthesis, subcellular targeting, or stability of cathepsin D. Brain tissues from homozygous A/A American Bulldogs had a significant decrease in cathepsin D activity to 36% of that in control dog brain tissue. It is unlikely that this activity reflects contributions from enzymes other than cathepsin D, because the identical assay procedure failed to detect any activity in brains from sheep homozygous for a cathepsin D missense mutation involving an active site aspartate [8]. Fifteen other lysosomal enzyme

activities in affected brains were either similar to normal (n=9) or significantly increased (N=6; see Table 3). This apparent compensatory upregulation of other lysosomal enzymes in response to a cathepsin D deficiency was also noted in brain tissue from the sheep with congenital NCL [8] and is typical of lysosomal storage disorders. In contrast to the NCLs of sheep and mice, which lack detectable cathepsin D activity [8,9], brains from homozygous affected American Bulldogs retained 36% of the cathepsin D activity found in control dog brains. This appears to be comparable to the reduction to 40% of normal cathepsin D activity, which was reported for fibroblasts cultured from clinically normal sheep that are heterozygous for the ovine CTSD mutation [8,18]. It is not clear that a reduction in an enzyme activity in brain sections from one species can be validly compared to a reduction in that enzyme activity in fibroblast cultures from another species. Furthermore, it is uncertain by what criteria or at what age the heterozygous sheep were evaluated for symptoms of disease. Nonetheless, it is surprising that the 36% residual cathepsin D activity did not protect the homozygous American Bulldogs from developing lethal NCL. It is possible that abnormal subcellular targeting or other aberrations prevent affected dogs from

fully benefiting from the residual cathepsin D activity detected in their brains. On the other hand, the clinical manifestations of American Bulldog NCL appear to be substantially less severe than those endured by the sheep or mice that completely lack cathepsin D activity. Thus, the residual cathepsin D activity in the dogs is likely to be of some benefit in moderating the symptoms. Human cathepsin D deficiency diseases have not yet been described. Characteristics of the cathepsin D deficiencies of sheep and mice suggest that the human disease might present as a congenital NCL. The American Bulldog disease indicates that human cathepsin D deficiency might also present as a later onset disease. Whether or not human cathepsin D deficiency diseases exist, the American Bulldog NCL may be a useful model for those developing gene or enzyme replacement therapies for lysosomal storage diseases affecting the brain.

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Disease Support and Research Association, and Research to Prevent Blindness,
Inc.

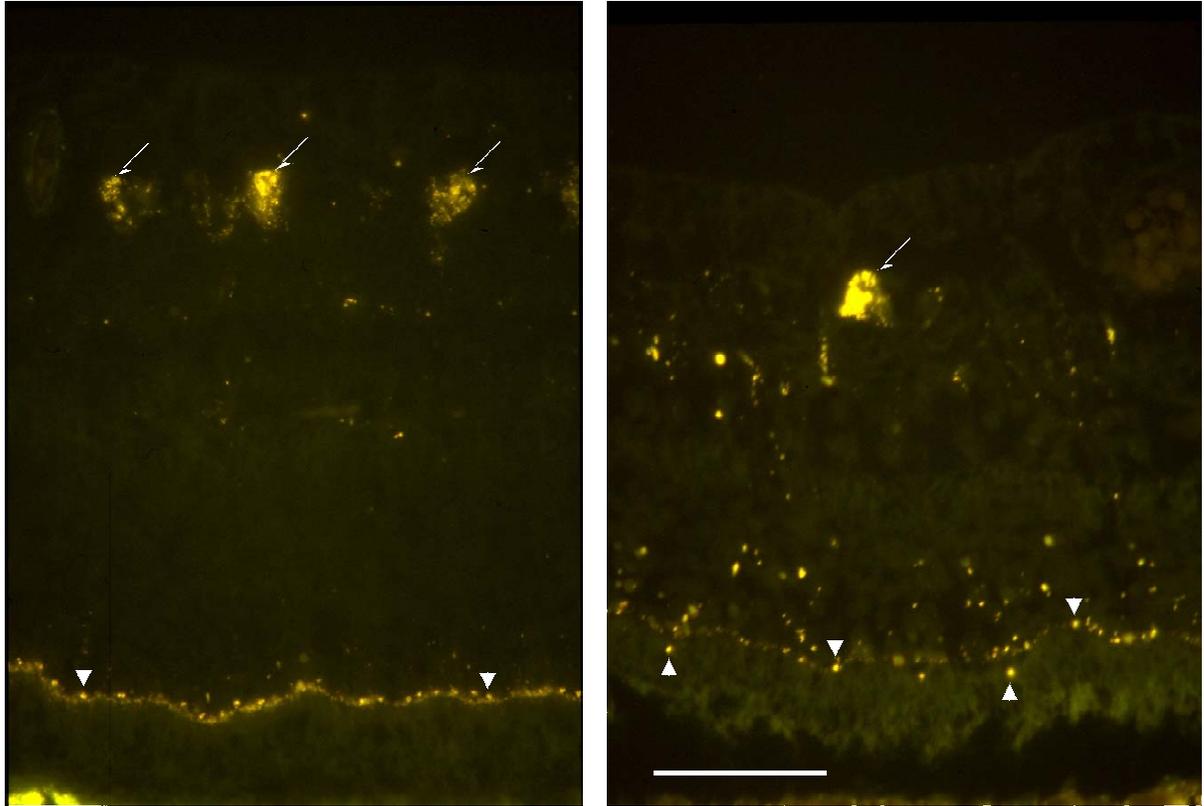


Fig. 1. Fluorescence micrographs of central (A) and peripheral (B) regions of the retina from an American Bulldog that was euthanized after exhibiting clinical signs of NCL. In the central retina, autofluorescent storage bodies were present primarily in the ganglion cells (arrows in A) and along the inner side of the outer limiting membrane (arrowheads in A). In the peripheral retina, storage bodies were present not only in the ganglion cells (arrow in B) and along the inner side of the outer limiting membrane (downward pointing arrowheads in B), but also on the photoreceptor inner segment side of the outer limiting membrane (upward pointing arrowheads in B). Bar in (B) indicates magnification for both micrographs.

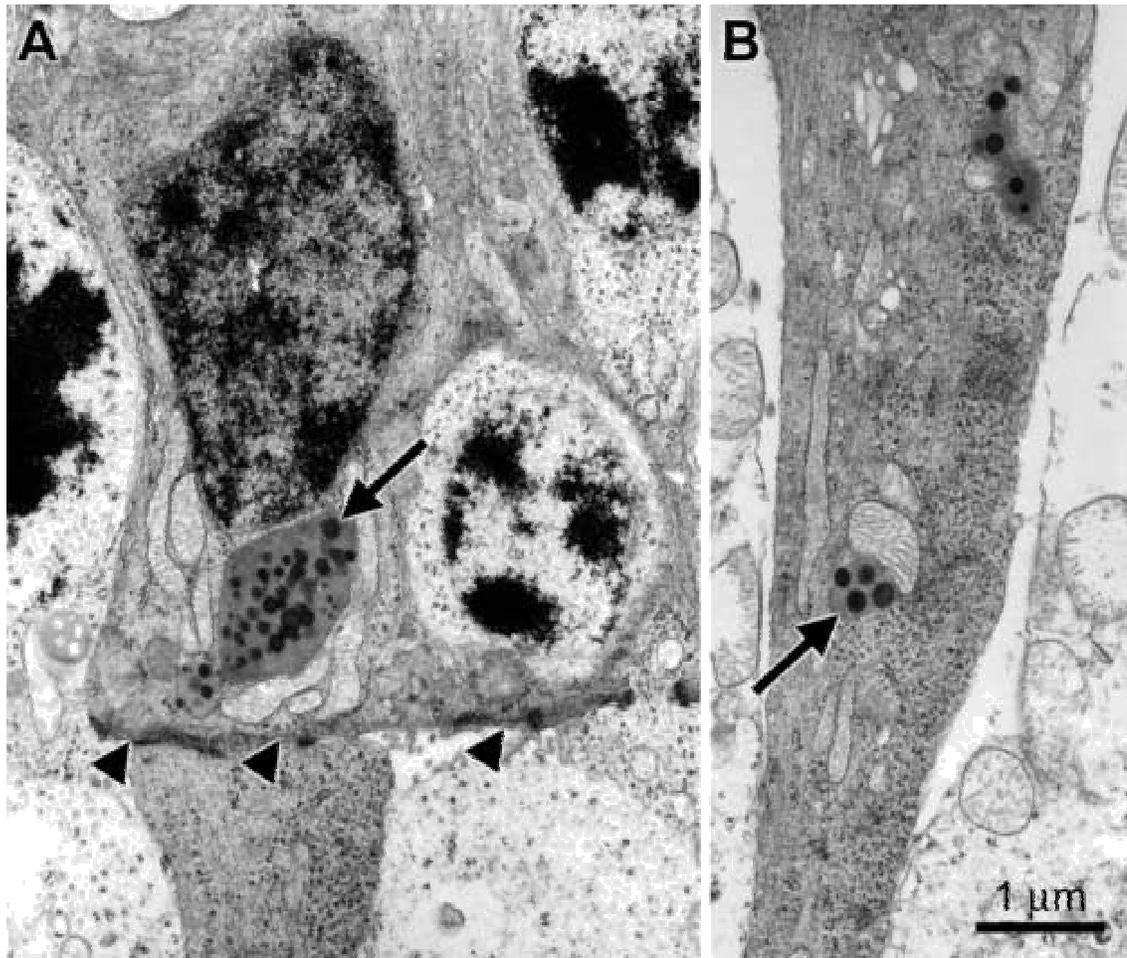


Fig. 2. Electron micrographs demonstrating the presence of disease-specific storage bodies in photoreceptor cells. Storage bodies (arrow in A) were found within photoreceptor cells just interior to the outer limiting membrane (arrowheads in A), as well as less frequently in the photoreceptor inner segments (arrow in B).

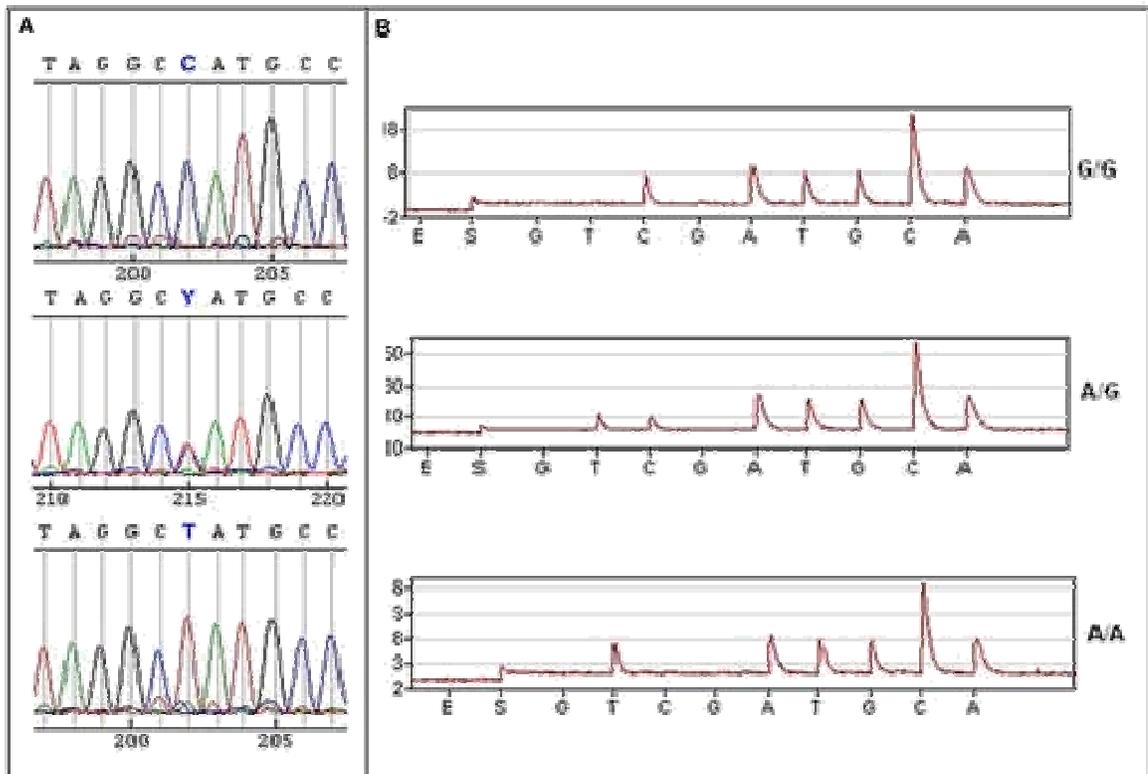


Fig. 3. (A) Aligned sequence chromatograms showing antisense sequence for a segment of *CTSD* exon 5 from an American Bulldog that is homozygous for the wild-type or G allele (top), from an A/G heterozygous American Bulldog (middle), and from a NCL-affected American Bulldog that is homozygous for the mutant or A allele (bottom). (B) Pyrosequograms produced in the antisense direction: from a G/G homozygote (top), an A/G heterozygote (center) and an A/A homozygote (bottom). See Ronaghi [22] for interpretation.

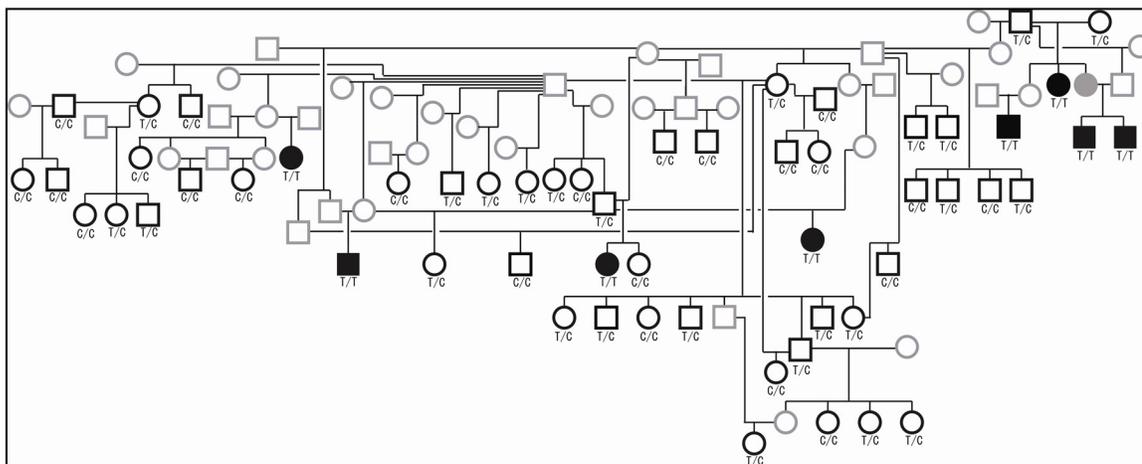


Fig. 4. Pedigree of an American Bulldog family segregating NCL: open squares and circles represent clinically normal individuals; solid squares and circles represent individuals that exhibited clinical signs of NCL. Genotypes at c.597 of CTSD are shown below each black symbol; DNA for genotyping was not available from the American Bulldogs represented in grey.

Table 1

Primers and conditions for amplification of canine *CTSD* exons

<u>Exon</u>	<u>Sequence 5'-3' (forward primer/reverse primer)</u>	<u>Annealing temp.</u>	<u>Amplicon size</u>
1 ^a	CCGGCTATAAGCGCACGACCT/CGTGGGACGCACACCGGGTCT	68 °C	366 bp
2	GGGAGCCCTGATGAACCCT/GCCCATACAGAACTTTCCTAA	58 °C	292 bp
3	AGCCTTAATGGACCCTGCTC/CCATAGTCTGCCTGCCTCCT	56 °C	203 bp
4	GCTGGCTGAGACCAAGGC/AGCCGTCTGAGCATCCC	58 °C	298 bp
5	TTGAGTCCTGGGTGCGTGGCCCTCT/AAAGCTGCCCGCTCGTCTG	63 °C	437 bp
6	GTGGCTGACTCCGCTTCT/CCTGCTCCCAAGGGCTGTA	58 °C	260 bp
7	CCTGAAGGAGGGGACTGC/CCTCTGAGGACAGCTTGTAAGT	58 °C	393 bp
8	GTGGACGAGGTGCGTGAGCT/CGCCTTGCGAAACCTG	58 °C	331 bp
9	CCTGCTGGACTCCTTCT/TGCTTACCACCCTGGCTAT	53 °C	227 bp

^aAmplification mixture for exon 1 contained 1M betaine.

Table 2

Amino acid sequences near methionine 199 of canine cathepsin D and its equivalent in cathepsin Ds from a variety of species

Amino Acid Sequence ^a	Species	Common Name
IAAKFDGILGMAYPRISVNNV	6 species of mammals ^b	
.....G.F..... ^c	<i>Rattus norvegicus</i>	Norwegian Rat
V.....G.H.....	<i>Mus musculus</i>	House Mouse
.....F....DK.	<i>Gallus gallus</i>	Chicken
.....DG.	4 fishes and a salamander ^d	
.....G.....DG.	<i>Tetraodon nigroviridis</i>	Green Spotted Pufferfish
.....A.DG.	<i>Silurus asotus</i>	Amur Catfish
.....FHIIA..	<i>Sparus aurata</i>	Gilt-head Seabream
V.....G.....DG.	<i>Xenopus laevis</i>	African Clawed Frog
V.....G.K...DG.	<i>Xenopus tropicalis</i>	Western Clawed Frog
.....L.....DK.	<i>Podarcis sicula</i>	Italian Wall Lizard
.....T...K.	<i>Todarodes pacificus</i>	Japanese Common Squid
.....T.A.DH.	<i>Bombyx mori</i>	Domestic Silkworm
.....NS...DK.	<i>Drosophila melanogaster</i>	Fruit Fly Species
.....SS...DG.	<i>Drosophila pseudoobscura</i>	Fruit Fly Species
V.....L..KS...DG.	<i>Anopheles gambiae</i>	African Malaria Mosquito
V.....LG.SS...DG.	<i>Aedes aegypti</i>	Yellow Fever Mosquito
.....F.E...LG.	<i>Ancylostoma ceylanicum</i>	Hookworm species
.....F.E.A.LG.	<i>Necator americanus</i>	New World Hookworm
VM.....SLA.GG.	<i>Schistosoma japonicum</i>	Blood Fluke species
VM.....S...DG.	<i>Schistosoma mansoni</i>	Blood Fluke species
.....KT...DG.	<i>Clonorchis sinensis</i>	Oriental Liver Fluke
V.....E.A.LG.	2 <i>Caenorhabditis</i> species ^e	

^aCathepsin D sequences corresponding to amino acids 189 to 209 in the dog protein; ^b*Canis familiaris* (Dog), *Homo sapiens* (Human), *Pongo pygmaeus* (Orangutan), *Bos Taurus* (Cow), *Ovis aries* (Sheep), *Sus scrofa* (Pig); ^cDot indicates same amino acid as dog and 5 other mammalian species; ^d*Oncorhynchus mykiss* (Rainbow Trout), *Clupea harengus* (Pacific Herring), *Takifugu rubripes* (Japanese pufferfish), *Chionodraco hamatus* (Antartic Icefish), *Hynobius leechii* (Korean Salamander); ^e*Caenorhabditis elegans*, *Caenorhabditis briggsae*.

Table.3

Enzyme	Specific activity ^a		
	Control brains ^b	NCL brains ^c	NCL/Control
<i>glycosidases</i>			
α -fucosidase	11.4 ± 2.2	20.2 ± 5.4	1.77 ^b
β -galactosidase	122 ± 27	157 ± 38	1.30
α -glucosidase	6.5 ± 0.7	11.9 ± 3.2	1.84 ^d
β -glucuronidase	45.4 ± 8.6	74.0 ± 14.2	1.67 ^d
β -hexosaminidase	271 ± 20	453 ± 18	1.73 ^d
β -hexosaminidase A	58.6 ± 7.3	101 ± 18	1.91 ^d
α -mannosidase	37.5 ± 4.3	48.7 ± 9.1	1.27
β -mannosidase	4.7 ± 0.6	5.2 ± 0.4	1.11
<i>peptidases</i>			
cathepsin B	14.7 ± 2.2	30.5 ± 11.9	2.07
cathepsin C	139 ± 13	119 ± 13	0.86
cathepsin D	2600 ± 263	927 ± 325	0.36 ^d
cathepsin H	195 ± 23	195 ± 15	1.00
dipeptidyl-peptidase II	19.7 ± 2.9	26.3 ± 4.7	1.33
legumain	11.1 ± 1.9	17.6 ± 6.4	1.60
tripeptidyl-peptidase I	57.4 ± 7.6	236 ± 25	4.11 ^d

^aEnzyme specific activities in canine brain tissues; ^bMean specific activities in 4 control brains ± S.D.; ^c Mean specific activities in 3 NCL-affected brains ± S.D.; ^dp < 0.05.

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Chapter V: A frame shift mutation in canine *TPP1* (the ortholog of human *CLN2*) in a juvenile Dachshund with neuronal ceroid lipofuscinosis

Abstract

The neuronal ceroid lipofuscinoses (NCLs) are inherited lysosomal storage diseases characterized by progressive neuropathy and the accumulation of autofluorescent cytoplasmic granules. Clinical signs of a new canine NCL began in a 9-month-old male Dachshund with vomiting, mental dullness, and loss of previously learned commands and rapidly progressed to include disorientation, ataxia, visual deficits, generalized myoclonic seizures, and death at 12 months of age. Neurons throughout the CNS contained autofluorescent storage granules that stained with periodic acid-Schiff and Luxol fast blue stains. Electron microscopy revealed that the storage granule contents consisted of curvilinear-appearing material characteristic of human late infantile NCL caused by *CLN2* mutations. Nucleotide sequence analysis of canine *TPP1*, the ortholog of human *CLN2*, revealed a single nucleotide deletion in exon 4 which predicted a frame shift with a premature stop codon. Brain tissue from the affected dog lacked detectable activity of the tripeptidyl-peptidase enzyme encoded by *TPP1*, whereas the specific activities of 15 other lysosomal enzymes were higher than

those in the brains of three control dogs. The affected Dachshund was homozygous for the mutant c.325delC allele, his sire and dam were heterozygotes, and 181 unrelated dogs, including 77 Dachshunds, were all homozygous for the wild type allele. A DNA assay that detects the mutant allele will help Dachshund breeders avoid producing affected puppies in future generations. Furthermore, this Dachshund NCL may prove to be a useful model for studying the pathogenesis of neurodegeneration in human late infantile NCL and for evaluating novel therapeutic interventions for this disease.

Introduction

The neuronal ceroid lipofuscinoses (NCLs) are heritable neurodegenerative diseases characterized by the accumulation of autofluorescent cytoplasmic storage granules in neurons and in other cell types. NCLs occur in people and in a variety of domestic and laboratory animals [1]. The human NCLs have been subclassified based on the age at onset, the patterns of symptoms, the ultrastructural appearance of the storage granule components, and the identity of the mutant gene responsible for the disease [2,3]. Mutations

in six genes, *PPT1*, *CLN2*, *CLN3*, *CLN5*, *CLN6*, and *CLN8*, have been reported to cause different forms human NCL [4-10]. In addition, different transgenic mice rendered nullizygous for one of four additional genes, *ctsd*, *clcn3*, *clcn7* and *ppt2*, develop NCL-like disorders [11-14].

Canine NCLs have been reported in at least 18 different breeds (http://www.caninegeneticdiseases.net/CL_site/basicCL.htm). These diseases are important both with respect to canine health and as potential models for the human NCLs. To date, the causative mutations have been identified in three breeds: the NCL of English Setters results from a missense mutation in *CLN8*; the NCL of Border Collies is due to a nonsense mutation in *CLN5*; and, the NCL of American Bulldogs is caused by a missense mutation in *CTSD* [15-17]. We here describe a new form of canine NCL that occurred in two juvenile Dachshund siblings and provide evidence that this disease resulted from a single base deletion in *TPP1*, the canine ortholog of human *CLN2*. This gene encodes the lysosomal enzyme tripeptidyl-peptidase 1 [18,19].

Materials and methods

Veterinarians in private practice provided medical records from two miniature longhaired Dachshunds, one male (the propositus) and one female littermate. The results of the physical examination, neurological examination, radiographs, and laboratory tests were reviewed. Videotapes of the propositus recorded at different stages of the disease were also viewed. The propositus died at one year of age and the remains were kept refrigerated for 36 hours prior to post-mortem tissue collection performed at the University of Pennsylvania. At necropsy, a portion of the tongue, the entire brain, and a portion of the spinal cord were removed. Small slices of the cerebellum, cerebral cortex and spinal cord were dissected from the samples and fixed in 10% buffered formalin. The remainder of the brain and the tongue were frozen. The fixed and frozen samples were shipped overnight to the University of Missouri. The tongue and brain tissues were then stored at -80°C until analyzed. A small portion of each fixed tissue was transferred to an electron microscopy fixative [15], another portion was processed for fluorescence microscopic examination [15], and the remainder was embedded in paraffin for light microscopic analyses.

Fluorescence and electron microscopy were performed as previously described [15]. Paraffin-embedded specimens were sectioned at 4 μm and standard procedures were used to stain them with hematoxylin and eosin, periodic acid-Schiff, and Luxol fast blue.

After a portion of the propositus' tongue had been macerated with a scalpel blade and digested for 48 hours at room temperature with protease K, the DNA was extracted as previously described [15]. In addition, DNA was extracted from blood samples from the sire and dam of the propositus. The University of Missouri Canine DNA Repository was the source of other DNA samples including all 77 Dachshund DNA samples in the collection and 104 samples selected to represent 104 other breeds.

The canine ortholog of human *CLN2* is annotated as "*TPP1*" in build 2.1 of the canine genome. *TPP1* occurs between 30108290 bp and 30114577 bp in CFA21 contig NW_876273.1 (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9615). PCR primer pairs flanking the coding regions of all 13 *TPP1* exons were designed from this sequence and used to amplify DNA from the propositus. The oligonucleotide sequences, annealing temperatures, and

predicted amplicon sizes for each primer pair are listed in Table 1. Amplicons were sequenced in both directions as previously described [15]. A PSQ™ 96 pyrosequencer was used to genotype the DNA samples with respect to a single-base deletion in *TPP1*. For pyrosequencing, the PCR primers were 5'-biotinyl-AAGTCTTGTGTGGTCACCGA-3' with 5'-TGACCTTCCGCACAG-3'; and, the sequencing primer was 5'-CTCAGCAGCTGGAGCC-3'.

Enzyme specific activities in cerebral cortex tissues from the propositus and from three normal dogs were determined as previously described [17]. Normal dog tissue samples were collected at the time of death and stored refrigerated for 36-48 hrs prior to freezing to duplicate the conditions under which the tissues from the propositus were handled. In addition, a second piece of cerebral cortex from one of the normal dogs was frozen immediately upon collection. Two separate pieces of each sample were homogenized and enzyme activity levels assayed separately for each piece. The duplicate values obtained from each sample were averaged.

Results

The first clinical signs of neurodegeneration in the propositus were noted at 9 months of age and included vomiting, mental dullness, loss of housebreaking, and unresponsiveness to previously learned commands. By 10 months of age, the dullness became worse, and the propositus developed ataxia and visual deficits. At 11 months of age, myoclonus of the head began and progressed to generalized myoclonic seizures which responded to intravenous diazepam. The dog had episodes of hyperactivity and howling. He began to exhibit aggressive behavior, a hypermetric gait, and incessant circling. Vomiting became more frequent and diarrhea subsequently developed. Urinalysis, CBC, serum total T4, serum biochemical profile, and pre- and post-prandial bile acids were within normal limits. A metabolic screen on freshly frozen urine for mucopolysaccharidosis and several other metabolic diseases was negative. Abdominal radiographs showed no apparent abnormalities. Treatment for potential encephalitis with enrofloxacin (3.9 mg/kg BID), prednisone (3.5 mg/kg/day) and vitamin supplements did not ameliorate the signs. The diarrhea progressed to hematochezia, and the dog died.

A female littermate of the propositus was ill from the time of weaning and slow to develop. She was diagnosed with demodecosis, and treated with oral ivermectin. At 7 and 8 months of age she had episodes of ataxia and disorientation which were originally attributed to ivermectin toxicity. Pre- and post-prandial serum bile acid concentrations at 7 months of age were within normal limits. This dog was examined for progressive ataxia, weakness and anorexia at 11.5 months of age. On neurological exam she was aggressive and appeared blind, but had a normal fundus. She was hypermetric and had a head tremor. Like her sibling, she died at 12 months of age. Tissues were not available from this female Dachshund.

Autofluorescent storage bodies were present in all examined regions of the central nervous system (Fig. 1). In the cerebral cortex, some cells had massive accumulations of these storage bodies, but the majority of cells contained little or no autofluorescent material (Fig. 1A). In the cerebellum, storage body accumulation occurred in all cell layers, but was more moderate in the Purkinje cells than in other cell types (Fig. 1B). In the spinal cord, the major accumulation of autofluorescent storage material occurred in the large motor

neurons (Fig. 1C).

Histopathological examination of the cerebellum at low magnification revealed a depletion of neurons in the cerebellar cortex and a narrowing of the molecular layer (Fig. 1F). The internal granular layer was very poorly populated. Purkinje cells were irregularly spaced along the junction between the molecular and internal granular layers with many atypically positioned in the granular cell layer. There was depletion of white matter. In the cerebrum and spinal cord, changes were less obvious at low magnification; however, at higher magnification most of the larger neurons in all specimens contained coarse eosinophilic cytoplasmic granules that were easily observed with periodic acid-Schiff and Luxol fast blue staining. The neuronal inclusions varied in number and size, but were well defined and polyhedral in shape (Fig. 1D-E).

Ultrastructural analyses indicated that the storage body contents in all of the neural tissues consisted almost exclusively of curvilinear forms (Figs. 2 and 3) characteristic of the lysosomal storage bodies that accumulate in association with human *CLN2* mutations [2,3]. Individual storage bodies from the proband were often quite large, measuring up to 7 μm in diameter. The storage bodies

clearly consisted of distinct organelles, although the surrounding membranes were often incomplete due to suboptimal fixation.

The ultrastructural similarity of the Dachshund storage granules to those that accumulate in human *CLN2* deficiency [2,3], prompted us to PCR-amplify and sequence all 13 canine *TPP1* exons from genomic DNA from the propositus and from an unrelated Dachshund. These sequences were compared to the Boxer DNA sequence produced in the canine genome project (<http://www.genome.gov/12511476>). The propositus' sequences from exons 1 to 3 and exons 5 to 13 were identical to the wild type Dachshund and Boxer sequences; however, exon 4 contained a single base pair deletion, c.325delC (Fig. 4A). This deletion alters the reading frame of the predicted messenger RNA after codon 109 and creates a premature stop codon at position 114.

We used a pyrosequencing assay capable of distinguishing wild-type homozygotes, mutant homozygotes, and heterozygotes (Fig 4B) to genotype DNA samples from the propositus, his sire and dam, and 181 other dogs including 77 unrelated Dachshunds and 104 dogs representing 104 other breeds. The propositus was homozygous for the c.325delC allele and his parents were

heterozygotes. The c.325delC allele was not detected in any of the other 181 unrelated dogs.

Tripeptidyl-peptidase 1 enzyme activity in the cerebral cortex from the propositus was less than 1% of the average activity in cerebral cortical tissue from the three control dogs (Table 2). In contrast, the specific activities of 15 other lysosomal enzymes in cerebral cortical tissue from the propositus were 33 to 284% higher than the mean specific activities in tissue from the three control dogs (Table 2). The 36 hours of refrigeration between death of the propositus and freezing of the tissue was unlikely to have substantially affected lysosomal enzyme activity levels, as similarly treated brain segments from a control dog yielded specific activities for all 16 lysosomal enzymes that were over 90% of the specific activities in segments from the same brain frozen shortly after death.

Discussion

Isolated reports of NCL in Dachshunds were published in the past. In 1977, Cummings and de Lahunta [20] described a wirehaired Dachshund with a

neurodegenerative disease that began with hind leg weakness at three years of age. The disease slowly progressed with marked cerebellar signs and a loss of the menace reflex with preservation of retinal function. Necropsy revealed cerebellar atrophy, yellow discoloration of cerebellar nuclei and the pontine nuclear area, and enlargement of the lateral and fourth ventricles. There was marked loss of cerebellar Purkinje cells with relative preservation of the granular cells. Cerebellar neurons and macrophages contained autofluorescent cytoplasmic granules. Electron microscopy revealed that the storage granules contained membranous forms, often arranged in parallel, forming zebra bodies.

In 1980 Vandeveld and Fatzer [21] described a neurodegenerative disease in two longhaired Dachshunds that exhibited mental deterioration at five and seven years of age. Microscopic examination of brains from these dogs revealed autofluorescent cytoplasmic inclusion in neurons throughout the brain. Within the cerebellum, there was marked degeneration of the granular cells with relative sparing of the Purkinje cells. Membraneous forms were prominent in the ultrastructure of the storage granules; however, fingerprint forms were common and zebra bodies were rare. These characteristics distinguish this latter

Dachshund disease from the earlier reported wirehaired Dachshund disease which appears to be identical to a more recently reported mucopolysaccharidosis IIIA of wirehaired Dachshunds caused by a three base pair deletion in the canine heparin sulfate sulfamidase gene [22,23].

The Dachshund disease described here differs from either of the two previously reported Dachshund diseases in that the current disease has a much earlier onset and is more rapidly progressive. Furthermore, the ultrastructural appearance of the storage bodies in the current case is quite distinct from those in the Dachshund diseases reported earlier. Instead of zebra bodies or fingerprint profiles, the storage body contents in the current case were almost exclusively curvilinear forms characteristic of the storage bodies from human late infantile NCL caused by mutations in *CLN2* [2,3].

This ultrastructural similarity prompted us to sequence the coding regions of *TPP1*, the canine ortholog of human *CLN2*, and led to the identification of a homozygous 1 bp deletion in exon 4 of *TPP1* in DNA from the propositus. This deletion causes a frame shift after 109 of the gene's 564 codons and predicts a premature stop codon at position 114. The premature stop codon

should lead to nonsense-mediated mRNA decay [24]. If any transcripts escape degradation in the pioneer round of translation, they would encode a markedly truncated protein lacking the catalytic serine required for enzyme activity [25]. Thus, it is not surprising that we failed to detect tripeptidyl-peptidase 1 enzyme activity in brain segments from the propositus. The high activities of other lysosomal enzymes in the affected brain were also anticipated as similar compensatory enzyme activity increases have been found in the brains of children with *CLN2* mutations [26] and in the brain of a dog with a mutation in *CTSD* [17]. Although the elevation in lysosomal enzyme activity levels in the affected dog varied from 33% to 284%, depending on the particular enzyme, the pattern did not appear to be specific for any functional class of lysosomal enzymes (Table 2).

The absence of the mutant c.325delC allele from 181 unrelated canine DNA samples, including 85 from Dachshunds, suggests that the mutant allele is uncommon even among Dachshunds. The two NCL-affected Dachshund littermates are registered with the American Canine Association. No ancestor appears more than once in the propositus' four-generation pedigree from this

registry. If this pedigree is accurate, the mutation, which was shown to be inherited from both heterozygous parents, must have originated at least five generations ago. Thus, the mutation may be common in certain lines of Dachshunds. The identification of the *TPP1* mutation and the availability of an assay that detects the mutant allele may help Dachshund breeders avoid producing additional dogs with this fatal neurodegenerative disease in future generations.

The world wide prevalence of human NCL caused by *CLN2* mutations is estimated at 0.6 to 0.7 per 10⁶ individuals with an incidence of approximately 0.46 per 10⁵ live births [2]. Patients typically begin to show clinical signs of epilepsy at 2 to 4 years of age. As the disease progresses patients endure ataxia, cognitive decline, myoclonus, loss of vision, and pyramidal and extrapyramidal signs [2,3]. Most patients die before they reach their middle teens [3]. There are no effective treatments. A *cln2*-knockout mouse that mimics features of human NCL has been created and is potentially useful for studying the pathogenic mechanisms underlying the neurodegeneration and for evaluating therapeutic strategies [27]. Because the Dachshund NCL described here could

serve as a large animal model, we are preparing to set up a breeding colony to provide affected puppies to investigators for assessing the safety and efficacy of novel therapies prior to human clinical trials.

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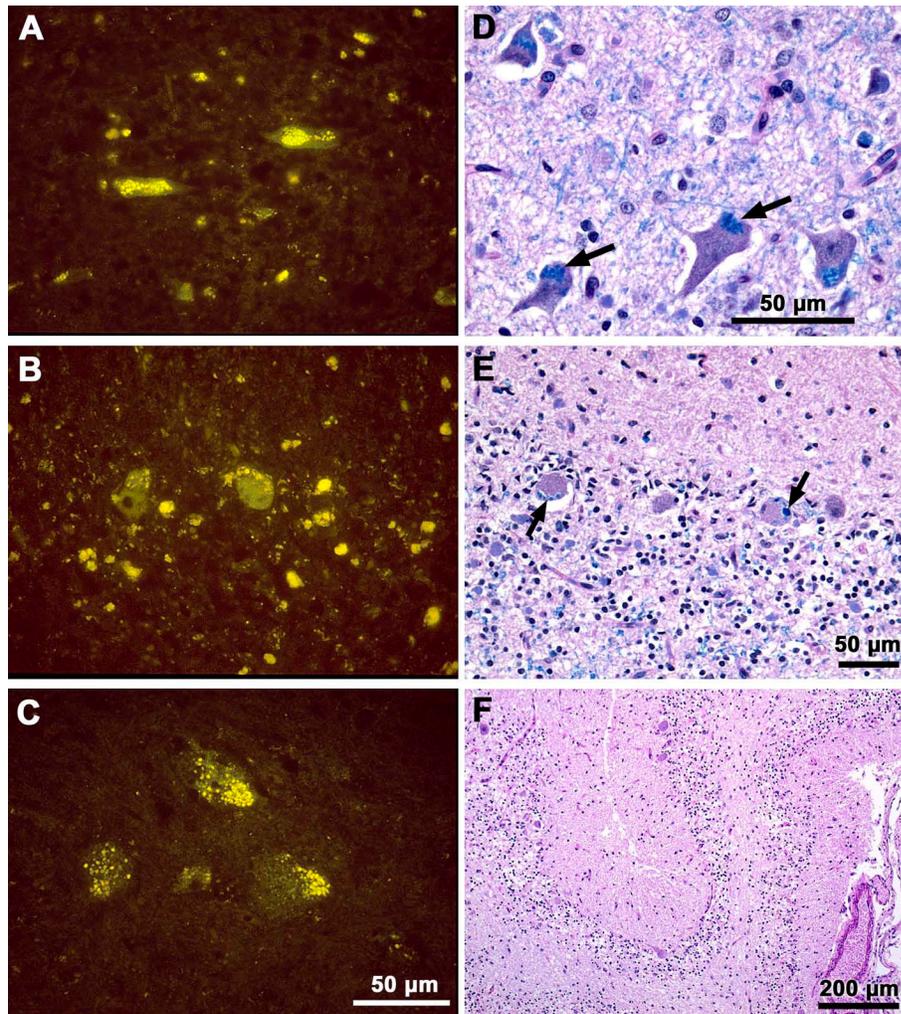


Fig. 1. Fluorescence micrographs of cryostat sections of cerebral cortex (A), cerebellum (B), and spinal cord (C) and sections of paraffin-embedded cerebral cortex (D) and cerebellum (E and F) from the propositus. Paraffin sections were stained with Luxol fast blue and counter stained with hematoxylin and eosin. Cellular inclusions that stained with Luxol fast blue were prominent in cerebral cortical neurons (arrows in A), cerebellar Purkinje cells (arrows in B) as well as in other cerebellar cells. Cell loss in the cerebellar cortex is apparent in F. Bar in (A) indicates magnification for (A), (B), and (C).

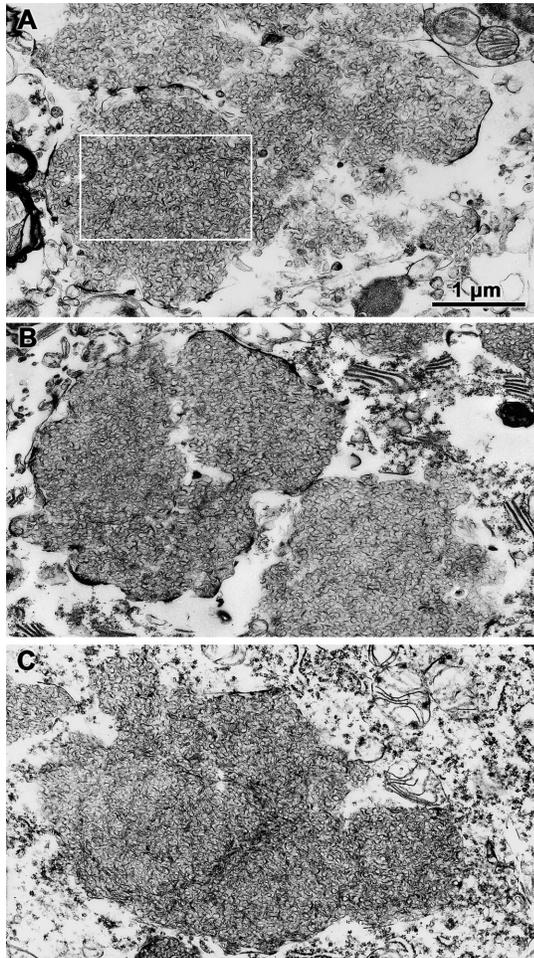


Fig. 2. Electron micrographs of storage bodies from the cerebral cortex (A), cerebellum (B), and spinal cord (C) of the propositus. Bar in (A) indicated the magnification of all three micrographs. Box in (A) indicates region that is shown at higher magnification in Fig. 3.

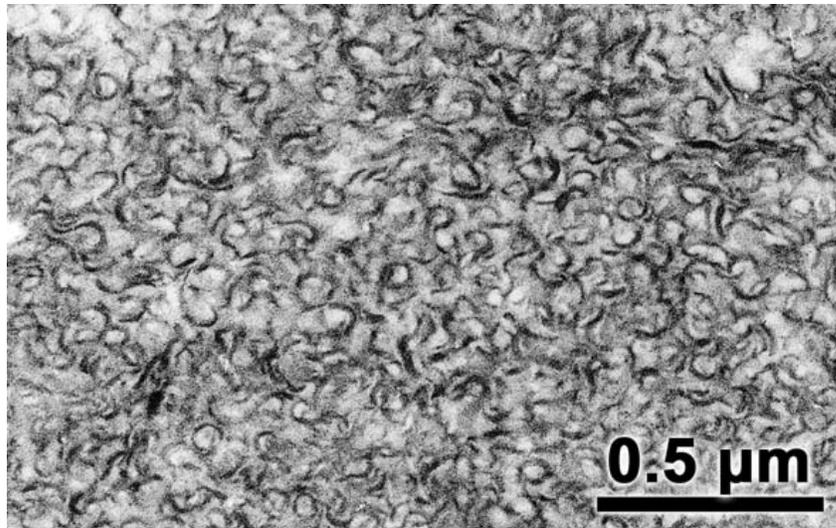


Fig. 3. Electron micrograph illustrating detailed ultrastructure of a portion of a cerebral cortex storage body shown in Fig. 2A.

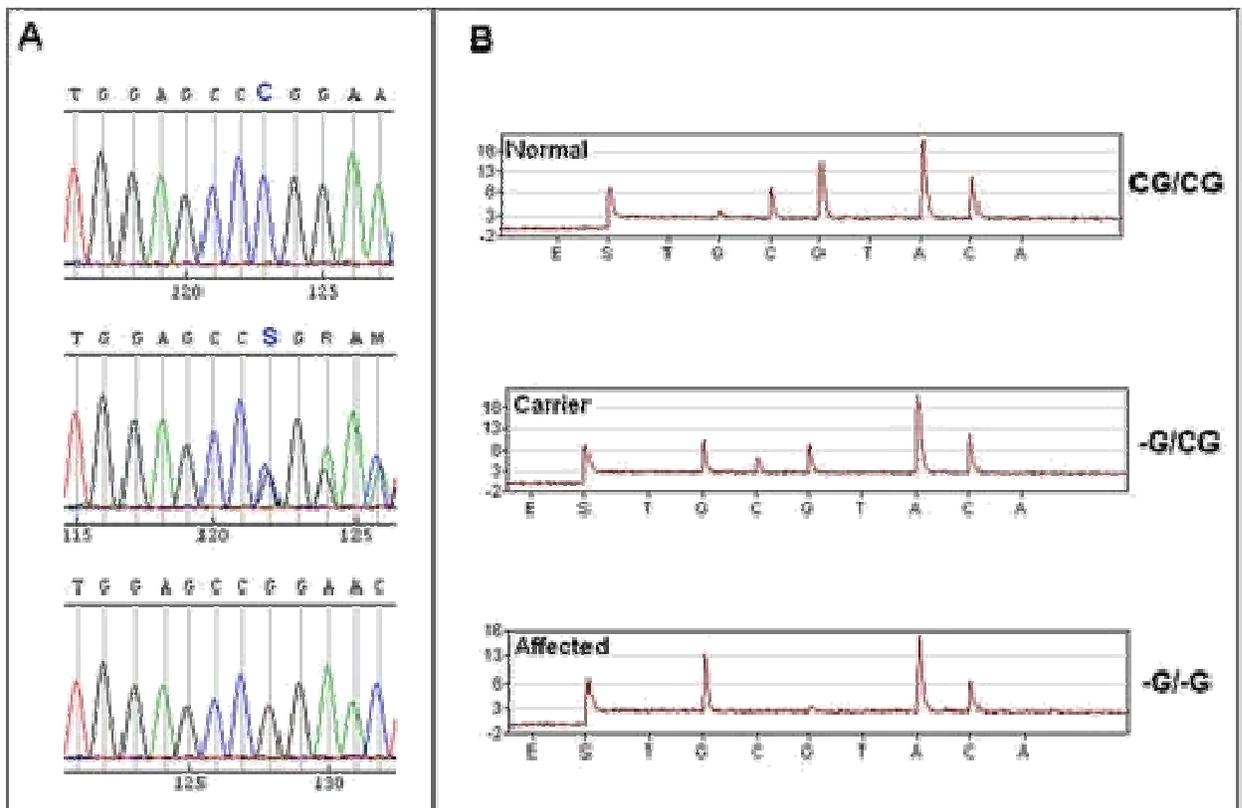


Fig. 4. (A) Aligned sequence chromatograms from a segment of *TPP1* exon 4 from a dog that is homozygous for the wild type allele (top), from the heterozygous sire of the propositus (middle), and from the propositus homozygous for the c.325delC allele (bottom). (B) Pyrosequograms produced with DNA from the same three dogs. See Ronaghi [28] for interpretation.

Table 1

Primers and conditions for amplification of canine *TPPI* exons

<u>Exon</u>	<u>Sequence 5' - 3' (forward primer / reverse primer)</u>	<u>Annealing temp.</u>	<u>Amplicon size</u>
1 & 2	TGGCGGTGGAACATAGG/AGCTGGTAATGGGGTGAAG	55°C	345 bp
3	CCTGATCCTGAACCTTCCA/AGCCCTGAGTCCCTCCA	55°C	281 bp
4	ATGTCTCCTGGTTGAGGTCC/CCCTGATGGGATGATTGG	55°C	267 bp
5	AGCTCTGGTCTGTA CTGCTCT/ACTCTGTCTGCTTCAACCCT	52°C	223 bp
6	AGTGGGAATTGGAGTCTAAA/GAGCAAGCCCAACCTG	52°C	297 bp
7	GGCTGCATCCTCTGACC/CCCTTTCCCCACCCAC	61°C	280 bp
8	GTGGGTGGGGAAAGG/GTGGGGTCAAAATGCC	52°C	360 bp
9	TGACATCCGAACCCAC/CAGCAACCAGAGCCAGT	52°C	272 bp
10	CCCTTGAGACCACCACA/CCCTCCTACCATCTATCCC	52°C	202 bp
11	GGATAGATGGTAGGAGGGAT/AGGAACAGGGACGGATG	55°C	346 bp
12	TCCAATCACTTTGAACCC/CAGGACCCCATCAGAAC	52°C	265 bp
13	ACAGGCAGCCCCAGAT/AGTTGAGGGTTCAACAGG	52°C	276 bp

Table 2. Lysosomal enzyme activities in brain samples from the affected and control dogs.

Enzyme	<i>Specific Activity*</i>			
	Control (n=3)		Affected	Affected/ Control
	Mean	SEM		
<i>glycosidases</i>				
α -fucosidase	21.5	1.5	56.9	2.64
β -galactosidase	250	23	338	1.35
α -glucosidase	10.4	1.0	15.5	1.49
β -glucuronidase	84	9.3	323	3.84
β -hexosaminidase	413	27	963	2.33
β -hexosaminidase A	7.7	0.3	16.7	2.17
α -mannosidase	62.3	10.6	121	1.94
β -mannosidase	4.4	0.6	11.1	2.52
<i>peptidases</i>				
cathepsin A	3103	327	4942	1.59
cathepsin B	98.5	4.5	172	1.75
cathepsin C	105	30	333	3.17
cathepsin D	3166	403	5498	1.74
cathepsin H	283	34	377	1.33
dipeptidyl-peptidase II	23.5	2.8	37.8	1.61
legumain	15.8	1.1	28.8	1.82
tripeptidyl-peptidase I	96.6	12	0.5	<0.01

* Specific activities are nmol substrate hydrolyzed/h/mg protein, except cathepsin D which is pmol substrated hydrolyzed/h/mg protein.

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Chapter VI: Further investigation of canine neuronal ceroid lipofuscinosis

NCLs are known as lysosomal dysfunction diseases. So far, all candidate genes encode lysosomal function are unknown [1-6]. We have found mutations in *CLN8*, *CTSD*, and *CLN2*.

Lonka et al [7] reported that the subcellular localization of the CLN8 protein was the ER and ER-Golgi intermediate compartment (ERGIC). In addition, CLN8 protein has a KKRP domain on its C-terminus which is an ER retrieval signal. Therefore, it was suspected that CLN8 is resident in ER and recycled between ER and ERGIC [7]. So far, it was not sure how CLN8 interacts with lysosomes. I would like to determine the location of CLN8 in cells from English Setters with NCL using immunolabeling under the confocal microscope. This may help establish how CLN8 protein interacts with membranes of the golgi apparatus.

Cathepsin D is known to be a major lysosomal enzyme. It is not difficult to think that the decreasing activity of cathepsin D might lead dysfunction of cell metabolism and might lead to cell death. However, the cathepsin D activity of affected American Bulldogs showed 35% of the activity in brains from normal

dogs. Cultured fibroblasts from asymptomatic heterozygous sheep had 50% of the cathepsin D activity in wildtype fibroblast cultures [8]. It was not sure that any threshold existed between 35% and 50% activity. And also, it was not sure that tissue enzyme activity and fibroblast cell enzyme activity could be compared equally. Cathepsin D is also distributed in the cytosol, but has unique characteristics. Cathepsin D only works as an aspartyl protease under acidic condition. Within the cell, the lysosome might be only place to provide an acidic environment for aspartyl protease activity. It was also unknown how much Cathepsin D is retained in lysosomes. The density gradient centrifugation could be used to fractionate subcellular components of cultured fibroblast and determine the distribution of Cathepsin D activity. Alternatively, the subcellular location of Cathepsin D could be determined using antibodies under the fluorescent microscope.

CLN2 encodes tripeptidyl peptidase I which removes tripeptides from the amino-terminus of peptides [9]. Ezaki et al. [10] revealed that CLN2 protein played an important role in the lysosomal degradation of subunit C of mitochondrial synthase, but the possibility still remains that another unknown

protease could participate in subunit C degradation. Subunit C is also found in other forms of NCLs (CLN3 and CLN5, and CLN8 gene mutations) [4, 11, 12]. Vesa et al. [13] said that CLN5 directly interacts with the CLN2 and CLN3 based on coimmunoprecipitation and an in vitro binding assay, but it is not clear what happens in vivo. Using live cell imaging, these protein interactions can be confirmed. Fluorescent Resonance Energy Transfer (FRET) might be a good way to investigate these interactions. CLN2 and CLN5 or CLN3 and CLN5 (labeled with green, blue, or yellow fluorescent protein.) could be cotransfect into cell cultures. These protein interactions could then be captured with the confocal microscope. Moreover, using a *CLN8* mutated cell line for FRET would help to verify that CLN8 has interaction with CLN5, CLN2, and CLN3 proteins. If this happens in vivo, it would indicate that CLN2, CLN3, CLN5, and CLN8 all contribute to subunit C degradation.

Canine NCLs have been recognized in at least 17 different breeds. Some of these are rare diseases like the human NCLs. However, in other breeds NCLs are quite common [14]. Because of the difficulty of early diagnoses and carrier detection, without appropriate breeding, potential risks are increasing. The

confirmation of storage bodies under the electron microscope or fluorescent microscope is the only way to make a definitive diagnosis, but this requires a brain biopsy. Diagnoses with DNA markers reduce potential risks. So far, English Setters, American Bulldogs, Border Collies, and Dachshunds are the only breeds for which DNA diagnostics are available [14][chapter 2-4]. We have been also working on Tibetan Terriers, Polish Lowland Sheepdogs, and Australian Shepherds NCLs. We have PCR-amplified and sequenced exons from candidate genes. The *CTSD* gene has been excluded in these three breeds. The *PPT2* gene has been excluded for Tibetan Terriers and Polish Lowland Sheepdogs. The *PPT1* gene has been excluded in Polish Lowland Sheepdogs. So far we haven't found any mutations which are associated with NCLs in these breeds. We are going to sequence the rest of the candidate genes to search for the mutations in affected dogs. However, this strategy will only work for mutations in coding regions or intron/exon splicing junctions, not for the promoter region. To find new target genes or mutations in promoter regions, we will apply linkage analysis to determine the chromosomal locations of the causative mutations.

Clark et al. [15] picked 327 canine microsatellite markers which have been multiplexed into chromosome-specific panels. Each marker covers an average 9 Mb of the canine genome with no gaps larger than 17.1 Mb. We will use these multiplexes, to genotype the all markers which will cover the whole genome. Then we will apply linkage analysis based on phenotype information. NCLs are heterogenic diseases. Even within the same breed, more than one form can occur [16, 17]. In this case, it is suspected that different genes cause different forms of NCL. Therefore, we will have to pay much attention to the phenotypic classifications and the pedigrees.

Some knockout mice have been made and are used as animal models for human NCLs [18-20]. However, it is difficult to assess disease phenotype in mice because of their small size and relatively primitive nervous systems. Therefore, dogs with NCLs are better than mice as animal models for human NCLs, especially in behavioral studies and therapeutic trials.

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