DOCTORAL THESIS

Study on the molecular bases of feline Pompe disease and Niemann-Pick disease type C

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Study on the molecular bases of feline Pompe disease and Niemann-Pick disease type C

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DEDICATION

I would like to dedicate this thesis to my late father, as well as my mother and wife, whose unwavering support and inspiration have motivated me to pursue higher education.

ABSTRACT

Lysosomal storage diseases refer to a collection of uncommon, inherited genetic disorders that affect cellular catabolism that usually arise from autosomal recessive inheritance and are caused by mutations or alterations in the coding sequence of acid hydrolases, or their activators located in the lysosome. These genetic defects lead to a decrease or complete loss of catalytic activity of the particular enzymatic reactions, causing an accumulation of the substrates of those reactions within the lysosome. Therefore, many lysosomal storage diseases present as progressively worsening, neurological conditions that can ultimately be fatal. Pompe disease (PD) and Niemann-Pick (NP) disease are rare genetic disorders found in cats that resemble their human counterparts. The elucidation of the genetic defects responsible for these diseases can aid in understanding their pathophysiology, development of new treatments and therapies, and potential benefits for human medicine.

Chapter 1: PD is an autosomal recessively inherited fatal genetic disorder that results from the deficiency of a glycogen hydrolyzing enzyme, acid α-glucosidase encoded by the *GAA* gene. Here, we describe the molecular basis of genetic defects in an 8-month-old domestic short-haired (DSH) cat with PD. The cat was previously diagnosed with PD based on the clinical and pathological findings of hypertrophic cardiomyopathy and excessive accumulation of glycogen in the cardiac muscles. Sanger sequencing was performed on 20 exons of the feline *GAA* gene using genomic DNA extracted from paraffin-embedded liver tissues. The affected cat was found to be homozygous for the *GAA*:c.1799G>A mutation resulting in an amino acid substitution (p.R600H) of acid α-glucosidase, a codon position of which is identical with three missense mutations (p.R600C, p.R600L, and p.R600H) causing human infantile-onset PD (IOPD). Several stability and pathogenicity predictors have also shown that the feline mutation is deleterious and severely decreases the stability of the GAA protein. The clinical, pathological, and molecular findings in the cat were similar to those of IOPD in humans. To our knowledge,

this is the first report of a pathogenic mutation in a cat. Feline PD is an excellent model for human PD, especially IOPD.

Chapter 2: NP disease type C (NPC) is an autosomal, recessive, and inherited neurovisceral genetic disorder characterized by the accumulation of unesterified cholesterol and glycolipids in cellular lysosomes and late endosomes, with a wide spectrum of clinical phenotypes. This study aimed to determine the molecular genetic alterations in two cases of felines with NP in Japan, a Siamese cat in 1989 and a Japanese domestic (JD) cat in 1998. Sanger sequencing was performed on 25 exons of the feline *NPC1* gene and 4 exons of the feline *NPC2* gene, using genomic DNA extracted from paraffin-embedded tissue specimens. The sequenced exons were compared with reference sequences retrieved from the GenBank database. The identified mutations and alterations were then analyzed using different prediction algorithms. No pathogenic mutations were found in feline *NPC1*; however, c.376G>A (p.V126M) was identified as a pathogenic mutation in the *NPC2* gene. The Siamese cat was found to be homozygous for this mutation. The JD cat was heterozygous for the same mutation, but no other exonic *NPC2* mutation was found. Furthermore, the JD cat had a homozygous splice variant (c.364-4C>T) in the *NPC2* gene, which is not known to be associated with this disease. The *NPC2*:c.376G>A (p.V126M) mutation is the second reported pathogenic mutation in the feline *NPC2* gene that may be present in the Japanese cat population.

In conclusion, the articles discussed the molecular basis of two different genetic disorders in cats. The first study that focused on PD identified a pathogenic mutation (*GAA*:c.1799G>A, p.R600H) in the feline *GAA* gene of a DSH cat with PD. This is the first report of a cat with PD carrying the same mutation as reported in a case of human classical IOPD. The clinical and histological findings in this cat with PD were similar to those in humans with IOPD. Therefore, this feline PD is an excellent model of human PD, especially IOPD. The second study that focused on NPC identified a pathogenic mutation (*NPC2*:c.376G>A (p.V126M)) in the feline

NPC2 gene of a Siamese cat and a JD cat with NP. The Siamese and JD cats were homozygous and heterozygous for this mutation, respectively. No other exonic *NPC2* mutation or deleterious splice variant was found in the JD cat, suggesting that this cat was a compound heterozygote of the identified *NPC2*:c.376G>A mutation and another pathogenic mutation that may be located in intronic regions. The c.376G>A (p.V126M) mutation is the second reported pathogenic mutation in the feline *NPC2* gene that causes NPC, and it may be present in the Japanese cat population. This feline models of PD and NPC may contribute to the development of new therapeutic strategies for treating those human diseases.

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FREQUENTLY USED ABBREVIATIONS

BLAST	-	Basic Local Alignment Search Tool
bp	-	base pair
c.	-	coding DNA sequence
ddG	-	delta delta G
DNA	-	deoxyribonucleic acid
FTA	-	flinders technology associates
GAA	-	acid alpha-glucosidase
GSD	-	Glycogen storage disease
IOPD	-	infantile-onset Pompe disease
LOPD	-	late-onset Pompe disease
MIA	-	mendelian inheritance of animal
MIM	-	mendelian inheritance of man
NA	-	not applicable
NCBI	-	National Center for Biotechnology Information
NP	-	Niemann-Pick disease
NPC	-	Niemann-Pick type C
p.	-	protein sequence
PCR	-	polymerase chain reaction
PD	-	Pompe disease
PDB	-	Protein Data Bank
SNP	-	single nucleotide polymorphism

CHAPTER 1

Investigation of genetic defects responsible for Pompe disease in a domestic short-haired cat

The above-titled work originally appeared in "*Animals* (Rakib et al., 2023) as: **Novel Mutation in the Feline *GAA* Gene in a Cat with Glycogen Storage Disease Type II (Pompe Disease)** authored by:

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1.1. ABSTRACT

Glycogen storage disease type II (Pompe disease: PD) is an autosomal recessively inherited fatal genetic disorder that results from the deficiency of a glycogen hydrolyzing enzyme, acid α-glucosidase encoded by the *GAA* gene. Here, we describe the molecular basis of genetic defects in an 8-month-old domestic short-haired cat with PD. The cat was previously diagnosed with PD based on the clinical and pathological findings of hypertrophic cardiomyopathy and excessive accumulation of glycogen in the cardiac muscles. Sanger sequencing was performed on 20 exons of the feline *GAA* gene using genomic DNA extracted from paraffin-embedded liver tissues. The affected cat was found to be homozygous for the *GAA*:c.1799G>A mutation resulting in an amino acid substitution (p.R600H) of acid α-glucosidase, a codon position of which is identical with three missense mutations (p.R600C, p.R600L, and p.R600H) causing human infantile-onset PD (IOPD). Several stability and pathogenicity predictors have also shown that the feline mutation is deleterious and severely decreases the stability of the GAA protein. The clinical, pathological, and molecular findings in the cat were similar to those of IOPD in humans. To our knowledge, this is the first report of a pathogenic mutation in a cat. Feline PD is an excellent model for human PD, especially IOPD.

1.2. INTRODUCTION

Glycogen storage disease type II (MIM # 232300), also known as Pompe disease (PD), is a rare autosomal recessive metabolic disorder caused by the deficiency of the lysosomal acid α-1,4 glucosidase (GAA, EC 3.2.1.20) (Seppälä et al., 2013; Bychkov et al., 2021). Because of GAA deficiency, glycogen accumulates in the lysosomes of many tissues, particularly cardiac and skeletal muscles (Kishnani et al., 2014; Almeida et al., 2017; Mokhtariye et al., 2019). The human *GAA* gene is located on chromosome 17q25.3 and is approximately 20 kb in size. The feline *GAA* gene is located on chromosome E1. It contains 20 exons, of which exon 1 is non-coding and codes for 952 amino acids in both humans and cats (Skelly and Franklin, 2002; Fukuhara et al., 2018).

The accumulation of glycogen in the lysosomes causes lysosomal enlargement and dysfunction, leading to cell damage and death. The damage and death of cells result in progressive muscle weakness and respiratory failure. The severity of the disease varies depending on the amount of residual GAA enzyme activity and the age of onset. In humans, PD can be classified as infantile-onset PD (IOPD) and late-onset PD (LOPD) (Fukuhara et al., 2018). In IOPD, the symptoms usually appear within a few months after birth and progress rapidly. The clinical symptoms include generalized muscle weakness, respiratory distress, hypertrophic cardiomyopathy, hypotonia, and macroglossia (Fukuhara et al., 2018; Thirumal Kumar et al., 2019). Widespread muscular glycogenosis, cardiac hypertrophy, and

hepatomegaly have been reported as pathological findings of IOPD (Skelly and Franklin, 2002; Dasouki et al., 2014; Smith et al., 2020; Tanaka et al., 2022). In contrast, the more common form, LOPD, the symptoms may not appear until adolescence or adulthood and progress more slowly. It has a heterogeneous presentation that occurs from childhood to late adulthood and is characterized by slowly progressive axial and/or limb-girdle muscle weakness, with or without respiratory symptoms. Sequestered respiratory failure, increased serum creatine kinase activity, atrial aneurysm, oropharyngeal dysphagia, ptosis, and scoliosis have also been reported in humans with LOPD (Dasouki et al., 2014; Almeida et al., 2017).

To date, more than 900 mutations in the human *GAA* gene have been reported in the PD GAA variant database (Niño et al., 2019; Niño et al., 2022). PD has also been described in other species, including dogs (OMIA 000419-9615) (Kishnani et al., 2012; Seppälä et al., 2013), cats (OMIA 000419-9685) (Sandström et al., 1969), cattle (OMIA 000419-9913) (Jolly et al., 1977; O'sullivan et al., 1981), sheep (OMIA 000419-9940) (Manktelow and Hartley, 1975), and the Japanese quail (OMIA 000419-93934) (Matsui et al., 1983). In dogs, a nonsense mutation, c.2237G>A (p.W746*), has been reported in Finnish and Swedish Lapphunds and was also reported at the same amino acid position in human IOPD (Seppälä et al., 2013). Three pathogenic mutations: c.1057_1058del (p.Y353L), c.1783C>T (p.R595*), and c.2454_2455del (p.T819R), have been reported in Brahman and Droughtmaster, Brahman, and Shorthorn cattle, respectively (Dennis et al., 2000). To date, no mutations have been identified in cats, quail, or

sheep with PD. Feline PD was first reported in a cat in 1969; however, the described pathological changes were limited to the brain because of the unavailability of liver and cardiac muscle specimens (Sandström et al., 1969; Seppälä et al., 2013). Molecular characterization was not performed at that time. The genetic defects underlying feline PD remain unknown.

Recently, our research group reported an eight-month-old domestic short-haired (DSH) cat with heart failure as another case of feline PD (Tanaka et al., 2022). This study aimed to identify the feline pathogenic *GAA* mutation in this cat with PD.

1.3. MATERIALS AND METHODS

This study was performed in accordance with the Guidelines Regulating Animal Use and Ethics of Kagoshima University (no. VM15041; approval date: 29 September 2015). Informed oral consent was previously obtained from the owner of the cat with PD (Tanaka et al., 2022).

Specimen

For DNA sequencing analysis, specimens were obtained from the paraffin-embedded liver samples from a DSH cat diagnosed with PD based on clinical, pathological, and ultrastructural findings (Tanaka et al., 2022). Genomic DNA was extracted from the specimens using automated extraction equipment (magLEAD 6gC, Precision System Science, Co. Ltd., Matsudo, Japan).

Mutation analysis

The coding exons and splice junctions of the feline *GAA* gene were amplified and assessed with polymerase chain reaction (PCR) and Sanger sequencing with specific primer pairs. The primer pairs were designed based on the reference sequence (XM_006940652.3) as the need arose because the DNA from the paraffin-embedded samples was modestly

fragmented (sequences in Appendix-I). PCR was performed in a 20 µL reaction mixture containing 10 µL 2X PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI, USA). The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions before sequencing. Sanger sequencing was performed by Kazusa Genome Technologies Ltd. (Kisarazu, Japan). The obtained sequencing data were compared with a reference sequence (XM_006940652.3) to identify candidate pathogenic mutations.

Pathogenicity and Stability Prediction

Pathogenicity and mutational changes in the stability of the predicted protein were analyzed using the PredictSNP, iStable, and FoldX servers, respectively. The PredictSNP server has an embedded algorithm for PredictSNP, MAPP, PhD-SNP, Polyphen-1, Polyphen-2, SIFT, SNAP, and Panther to classify the variants as 'deleterious' or 'neutral' (Bendl et al., 2014). Similarly, iStable has an embedded algorithm of iStable, i-Mutant, and MUpro to classify the variants as 'increasing' the stability and 'decreasing' the stability of the protein (Chen et al., 2013). Additionally, the empirical protein design forcefield FoldX was used to calculate the difference in free energy of the mutation: delta delta G (ddG). Stability was predicted based on the ddG value. If a mutation destabilizes the structure, ddG increases, whereas stabilizing

mutations decrease ddG. Since the FoldX error margin is around 0.5 kcal/mol, changes in this range are considered insignificant (De Baets et al., 2012). The amino acid sequences in the FASTA format and mutations were used as inputs for prediction.

PCR-Restriction Fragment Length Polymorphism (RFLP)

The PCR-RFLP was carried out with forward (5′-AGGGCCCTGGTCAAGGC-3′) and reverse (5′-TGACAGGCGCTCTCACCT-3′) primers in a 20 μL reaction mixture containing 10 μL 2X PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp), 12.5 pmol of each primer and extracted DNA of the affected cat and clinically healthy control cats as templates. After the first denaturation at 95 °C for 2 min, 40 cycles of amplification were carried out at an annealing temperature of 58 °C for 30 s and a final extension at 72 °C for 2 min. The PCR products were digested with the restriction enzyme *AciI* (New England Biolabs, Ipswich, MA, USA) at 37 °C for 1 h in a 10 μL reaction mixture containing 8 μL of PCR product, 10 U of *AciI*, and 2 μL of 10X restriction enzyme buffer. The digested PCR products were then visualized with agarose gel electrophoresis using 3% agarose gel using a molecular size marker (Marker 9, Nippon Gene Co. Ltd., Tokyo, Japan). One hundred DNA samples from mixed-breed cats, which were previously stored in the laboratory (Chang et al., 2012), were genotyped using PCR-RFLP.

1.4. RESULTS

Mutation Detection

Sanger sequencing was performed on 20 exons and splice junctions of the *GAA* gene in the cat with PD. The sequenced exon and splice junctions were compared to the reference sequence (XM_006940652.3). As a result, the sequencing and comparison revealed 21 homozygous coding, two heterozygous coding, one splice junction, two intronic, and two untranscribed region (UTR) variants (Figure 1). The coding variants included 10 homozygous missense mutations (c.55G>A, c.181G>A, c.1298G>A, c.1480G>A, c.1779G>A, c.1889A>G, c.2152G>T, c.2207G>A, c.2423C>G, and c.2629G>A), one heterozygous missense (c.2728G>A), and 12 silent mutations (c.72T>C, c.840C>T, c909C>T, c.1200G>T, c.1350C>T, c.1560A>G, c.1710T>C, c.1779G>T, c.2178T>C, c.2304C>T, c.2373C>T, and c.2688G>A).

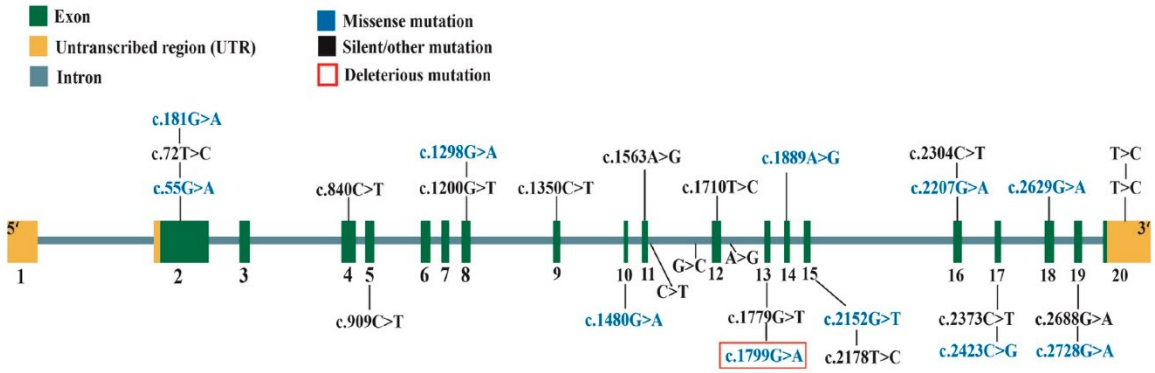

Figure 1: Identified variants in the *GAA* gene of the cat with Pompe disease. There were 21 homozygous coding, 2 heterozygous coding, 1 splice junction, 2 intronic, and 2 untranscribed region variants. The coding variants include 10 homozygous missense, 1 heterozygous missense mutation, and 12 silent mutations.

Pathogenicity and Stability Prediction

The 11 missense mutations were further analyzed using the SIFT and PredictSNP servers. One of the eleven identified missense mutations (c.1799G>A, p.R600H, Figure 2) showed a deleterious effect on the catalytic site of the GAA protein in the analysis of all eight pathogenicity predictors (Table 1). The SIFT prediction server also supported the same amino acid mutation (p.R600H) in exon 13 as deleterious, with a SIFT score (0.00). Another candidate mutation (c.55G>A, p.V19M) in exon 2 was predicted to be deleterious by five predictors with a moderately low SIFT score (0.03). These two predicted deleterious mutations were subjected to the iStable server to measure the change in the stability of the GAA protein upon mutation using the i-mutant 2.0, MUpro, and iStable algorithms (Table 2). Only one mutation (c.1799G>A, p.R600H) in exon 13 was predicted to decrease the stability of the GAA protein by all three stability predictors.

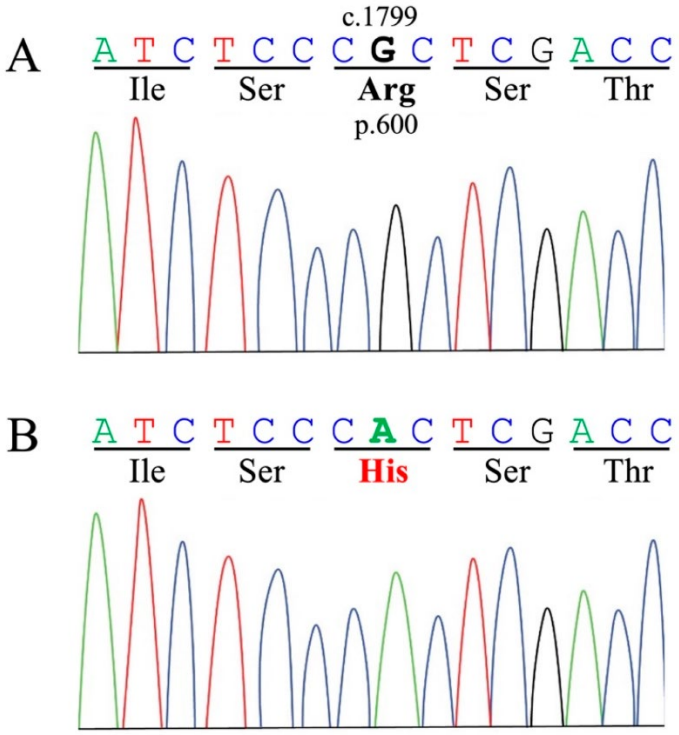

Figure 2. Comparison of sequenced data of the affected cat with the reference data in exon 13 of the feline *GAA* gene (c.1799G>A, p.R600H). The Sanger sequencing for a wild-type homozygous genotype (A) and a mutant homozygous genotype ((B), the affected cat) are shown.

To date, no model for the feline GAA protein crystal structure is available in the Protein Data Bank (PDB) database. Therefore, we performed a protein-protein BLAST analysis between the reference feline GAA protein sequence and the PDB database at the National Center for Biotechnology Information (NCBI) server to predict identical proteins. An identical human GAA protein (PDB ID: 5KZW, query coverage: 91%) was predicted and used to predict the stability in the FoldX predictor because of the unavailability of a feline GAA protein model in the PDB database. After FoldX prediction, the mutation from arginine to histidine at amino acid position 600 resulted in a ddG of 12.62 kcal/mol. This implied that this mutation

(p.R600H) severely reduced GAA protein stability (Figure 3). However, the stability effect of the p.V19M mutation could not be predicted because of the absence of the first 79 amino acids in the reference protein model. Overall, the mutation c.1799G>A (p.R600H) in exon 13 was predicted to be deleterious and to decrease the stability of the GAA protein by all pathogenicity and stability predictors used in this study.

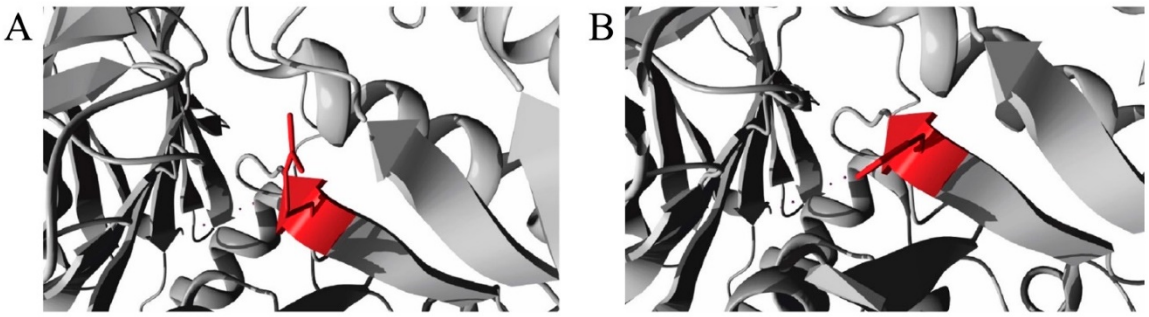

Figure 3. Molecular visualization of the wild-type (A) and the p.R600H mutant variant (B) of the amino acid in the feline GAA protein. The residues colored in red represent the wild-type (arginine) and variant residue (histidine) residues.

Table 1. Analysis of missense mutations in the catalytic site of the GAA protein using the SIFT and PredictSNP servers.

Exon No.	Mutations	SIFT Score	SIFT	PredictSNP	MAPP	PhD-SNP	Polyphen-1	Polyphen-2	SNAP	PANTHER
2	V19M	0.03	D	D	UK	N	D	D	D	N
2	G61S	0.92	N	N	N	N	N	N	N	UK
8	R433Q	0.91	N	N	N	N	N	N	N	N
10	E494K	0.55	N	N	N	N	N	N	N	N
13	R600H	0.00	D	D	D	D	D	D	D	D
14	E630G	0.39	N	N	N	N	N	N	N	N
15	V718L	0.32	N	N	N	D	N	N	N	N
16	R736H	0.06	N	N	N	N	N	N	N	N
17	P808R	0.24	N	N	N	N	N	N	N	N
18	V877I	0.21	N	N	N	N	N	N	N	N
19	A910T	0.60	N	N	N	N	N	N	N	N

D: deleterious; N: neutral; UK: unknown.

Table 2. Classification of two candidate deleterious missense mutations in the catalytic site of GAA protein using iStable server.

Exon No.	Mutations	i-Mutant 2.0	ddG	MUpro	Conf. Score	iStable	Conf. Score
2	V19M	Decrease	−1.81	Null	Null	Decrease	0.570209
13	R600H	Decrease	−1.63	Decrease	−0.30256	Decrease	0.685774

PCR-RFLP and Genotyping

Using the PCR-RFLP, stored control DNA samples from 100 clinically healthy cats were genotyped. All control samples were homozygous for the wild-type genotype (c.1799G/G), whereas only the cat with PD was homozygous for the mutant genotype (c.1799A/A) (Figure 4). We performed Sanger sequencing on five control DNA samples randomly selected from among 100 clinically healthy cats and found that three homozygous wild-type (c.55G/G), two heterozygous (c.55G/A), and one homozygous mutant (c.55A/A) genotypes.

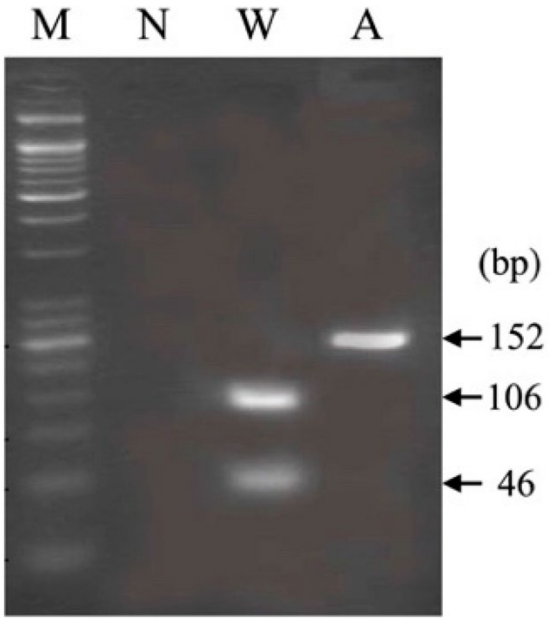

Figure 4. Genotyping of the wild-type (W) and affected (A) cats by polymerase chain reaction-restriction fragment length polymorphism using agarose gel electrophoresis. Lanes M and N show molecular size markers and non-template control, respectively.

1.5. DISCUSSION

We reported an eight-month-old DSH cat with heart failure as the second case of feline PD in 2022 (Tanaka et al., 2022), after the first case was reported in 1969 (Sandström et al., 1969). The diagnosis in our cat was established based on clinical and pathological characteristics similar to those of human PD (Tanaka et al., 2022). Our cat was first presented at eight months of age with acute tachypnea, poor growth, hypothermia, and lethargy at a veterinary hospital after a three-month history of unexplained fever. Radiography revealed that the cat had cardiomegaly. Echocardiography revealed dilatation of both atria and left ventricular systolic dysfunction. The cat died of pulmonary edema caused by chronic heart failure. The severe clinical manifestations in the cat resembled those of human IOPD. Postmortem histological examination revealed severe vacuolation of the cardiac muscle cells stained with hematoxylin and eosin. Periodic acid-Schiff staining positively stained coarse granules within the vacuoles that disappeared upon pre-digestion with diastase, indicating that glycogen accumulated in the cardiac muscle cells. To identify the first pathogenic mutation in the feline *GAA* gene, we molecularly analyzed the genomic DNA from this cat with PD because the molecular basis of the genetic defect in feline PD remains unknown.

Sanger sequencing of the feline *GAA* gene of the affected cat and comparison with the reference sequence was performed to identify candidate pathogenic mutations in feline PD. As a result, 10 homozygous and 1 heterozygous missense mutations were found in the coding

region of the feline *GAA* gene (Figure 1). After the evaluation of pathogenicity and stability prediction (Table 1 and Table 2 and Figure 1 and Figure 3), two missense mutations, c.1799G>A (p.R600H) and c.55G>A (p.V19M), were selected as the most likely pathogenic mutations in the cat with PD. Based on the data obtained from several stability and pathogenicity predictors, the c.1799G>A mutation was more likely to be a pathogenic mutation. Furthermore, a preliminary genotyping survey strongly suggested that c.1799G>A is a pathogenic mutation in feline PD. That is because there were no cats with the c.1799G>A mutation in the 100 clinically healthy cats, whereas 2 heterozygous and mutant homozygous cats for the c.55G>A mutation were easily found in several cats from among the healthy cat population. This preliminary survey indicated that the c.1799G>A mutation was rare, suggesting that c.1799G>A might be pathogenic. In contrast, the c.55G>A mutation was not rare, and even homozygote (c.55A/A) was present without clinical symptoms among the five randomly selected clinically healthy cats. This suggested that c.55G>A was not related to feline PD with severe symptoms. However, a large-scale survey would be beneficial in detecting more cats carrying the c.1799G>A mutation and understanding the association of the c.55G>A mutation with feline cardiac function.

In human PD, arginine at amino acid position 600 is thought to be important for maintaining the function of the GAA protein because there are reports of missense mutations in which arginine at codon 600 is substituted with different amino acids, causing classical

human IOPD (Ko et al., 1999; Tsujino et al., 2000; McCready et al., 2007; Niño et al., 2019; Reuser et al., 2019; Niño et al., 2022). These were c.1798C>T (p.R600C), c.1799G>T (p.R600L), and c.1799G>A (p.R600H) that is the same as the feline PD mutation identified in the present study. The clinical manifestations of feline PD were as severe as those in human IOPD, and feline PD caused by c.1799G>A (p.R600H) was similar to human IOPD. In human IOPD, myocardial hypertrophy due to glycogen accumulation is likely to progress to hypertrophic and dilated cardiomyopathy (Tarnopolsky et al., 2016). Therefore, both the clinical signs and histopathological findings in our cat suggest that this feline PD is an infantile form of feline PD, similar to human IOPD. There may be feline PD cases with moderate or mild cardiac disorders caused by different GAA mutations in the cat population.

Enzyme replacement therapy (ERT) is currently the only effective treatment of PD (Kishnani et al., 2007; Van der Ploeg et al., 2010). Recent studies have shown that ERT extends the survival of both children and adults. ERT has been shown to improve ventilator-free survival even in patients with IOPD. However, it is expensive because of the requirement of a large dose of GAA enzyme therapy. Furthermore, a complete cure was not evident with the present form of ERT. Therefore, improving ERT or introducing alternative therapeutic interventions, such as gene therapy, is still needed in the near future. Given the current promising achievements in the field of gene therapy, this approach deserves further investigation for the treatment of PD. The feline PD model could be instrumental in future

therapeutic research as a large animal model of PD.

CHAPTER 2

Molecular investigation of genetic defects responsible for Niemann-Pick type C disease in a Siamese and a Japanese domestic cats

The above-titled work originally appeared in "*Animals* (Rakib et al., 2023)" as: **Novel Mutation in the Feline *NPC2* Gene in Cats with Niemann-Pick Disease** authored by:

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2.1. ABSTRACT

Niemann-Pick disease (NP) type C is an autosomal, recessive, and inherited neurovisceral genetic disorder characterized by the accumulation of unesterified cholesterol and glycolipids in cellular lysosomes and late endosomes, with a wide spectrum of clinical phenotypes. This study aimed to determine the molecular genetic alterations in two cases of felines with NP in Japan, a Siamese cat in 1989 and a Japanese domestic (JD) cat in 1998. Sanger sequencing was performed on 25 exons of the feline *NPC1* gene and 4 exons of the feline *NPC2* gene, using genomic DNA extracted from paraffin-embedded tissue specimens. The sequenced exons were compared with reference sequences retrieved from the GenBank database. The identified mutations and alterations were then analyzed using different prediction algorithms. No pathogenic mutations were found in feline *NPC1*; however, c.376G>A (p.V126M) was identified as a pathogenic mutation in the *NPC2* gene. The Siamese cat was found to be homozygous for this mutation. The JD cat was heterozygous for the same mutation, but no other exonic *NPC2* mutation was found. Furthermore, the JD cat had a homozygous splice variant (c.364-4C>T) in the *NPC2* gene, which is not known to be associated with this disease. The *NPC2*:c.376G>A (p.V126M) mutation is the second reported pathogenic mutation in the feline *NPC2* gene that may be present in the Japanese cat population.

2.2. INTRODUCTION

Niemann-Pick disease (NP) encompasses a group of autosomal recessive lysosomal storage disorders characterized by the accumulation of diverse lipid species in lysosomes (Torres et al., 2017). NP types A (infantile neurovisceral type, MIM #257200) and B (chronic visceral type, #607616) are caused by deficits in the activity of acid sphingomyelinase, an enzyme that regulates lysosomal sphingomyelin homeostasis, which is encoded by the *SMPD1* gene. NP type C (NPC) is caused by mutations in the *NPC1* (#257220) and *NPC2* genes (#607625), resulting in functional defects in lysosomal proteins NPC1 and NPC2, which are involved in cholesterol efflux from lysosomes.

NPC is an inherited neurovisceral genetic disorder characterized by the accumulation of unesterified cholesterol and glycolipids in cellular lysosomes and late endosomes, with multiple clinical phenotypes (Roszell et al., 2013). NPC is divided into two types of diseases, C1 and C2, based on the mutations in *NPC1* or *NPC2*. Both NPC proteins, NPC1 and NPC2, are required for sterol homeostasis and are thought to be responsible for sterol integration into the lysosomal membrane before its redistribution to other cellular membranes (Winkler et al., 2019). A soluble intralysosomal protein, NPC2, can transfer sterols to the luminal N-terminal domain of the lysosomal membrane protein NPC1 with reversed polarity by binding to sterols with distinct polarity (Lu et al., 2015). The intracellular trafficking of lipids such as cholesterol, lipophilic molecules, amines, and mycolic acids is mediated by NPC1 and NPC2 proteins (Wheeler and Sillence, 2020). Because of the functions of these proteins, when one or both are lacking or unstable, the lysosome/late endosome compartment begins to accumulate endocytosed unesterified cholesterol, gangliosides, and other lipids (Zampieri et al., 2014; Winkler et al., 2019; Torres et al., 2021). Therefore, NPC disorders stem from mutations in *NPC1* and *NPC2*, which affect quality control mechanisms involving the lysosome and endoplasmic reticulum (Torres et al., 2021).

In humans diagnosed with NPC, 133 *NPC1* and 11 *NPC2* pathogenic variants have been described (Vanier, 2015; Vanier et al., 2022). Because of the functions of these genes in lysosomes and late endosomes, the predominant biochemical characteristic of NPC is the lysosomal accumulation of unconjugated cholesterol and glycosphingolipids in the brain, liver, and other visceral organs (Torres et al., 2017; Torres et al., 2021; Vanier et al., 2022). Mitochondrial cholesterol buildup and dysfunction are also observed, which results in the impaired transport of cytosolic reduced glutathione (GSH) into the mitochondria. Moreover, a decreased level of mitochondrial GSH causes oxidative stress in the cells, and neurons and hepatocytes may die as a consequence of the loss of antioxidants, such as GSH (Torres et al., 2021). Therefore, neurovisceral cholesterol storage with progressive neuronal cell loss, axonal spheroids, and ectopic neurites are key microscopic findings in NPC. Clinical manifestations include progressive neurological symptoms such as dysmetria, ataxia, and generalized tremor, as well as significantly higher levels of unconjugated cholesterol in neurovisceral organs (Roszell et al., 2013; Vanier, 2015). The phenotypes of human NPC vary greatly, and manifest from fetal life to adulthood. Most patients experience a progressive and deadly neurological condition. However, an exception of this is found in those who have the perinatal form of the disease, which commonly results in death and appears at birth or during the first month of life with significant visceral involvement, including fetal hydrops, ascites, neonatal cholestasis, liver failure, and/or pulmonary disease (Brown et al., 1994; Zampieri et al., 2014).

The antemortal diagnosis of inherited genetic diseases can be a time-consuming and costly process due to the clinical similarities and progression of these diseases. Many genetic disorders are caused by cellular mechanics being depleted or altered, highlighting the need for a reliable and cost-effective molecular technique to identify the underlying cause of the disease for accurate diagnosis and management. A widely accepted approach in the study of genetic diseases is the use of polymerase chain reaction and sequencing, either targeting suspected

disease-related genes or employing genome-wide sequencing (Somers et al., 2003; Mauler et al., 2017; Vanier et al., 2022; Rakib et al., 2023). However, a significant challenge in the diagnostic process is predicting the pathogenicity of identified alterations in the genetic sequence. Over the past decade, numerous pathogenicity and stability predictors have been developed, particularly in the field of human genetic research (Chen et al., 2013; Bendl et al., 2014; Shibata et al., 2016; Chen et al., 2020). These prediction tools utilize various factors related to pathogenicity, but no single algorithm can provide absolute accuracy in mutation prediction. Therefore, making decisions based on a mutation can be challenging. To address this, many researchers recommend combining the results from different prediction algorithms to arrive at a final decision (Chen et al., 2013; Bendl et al., 2014; Sanavia et al., 2020).

NPC has also been described in animals, including a Boxer dog (Type C1, OMIA 000725-9615) (Kuwamura et al., 1993), cats (Type C1, OMIA 000725-9685; Type C2, OMIA-002065-9685) (Somers et al., 2003; Vite et al., 2008; Zampieri et al., 2014; Mauler et al., 2017), and Angus cattle (Type C1, OMIA 000725-9913) (Woolley et al., 2020; Woolley et al., 2021). In cats, two pathogenic mutations, c.2864G>C (p.C955S) (Somers et al., 2003) and c.1322A>C (p.H441P) (Mauler et al., 2017), in the feline *NPC1* gene, and one pathogenic mutation, c.82+5G>A (p.G28_S29ins35) (Mauler et al., 2017), in the feline *NPC2* gene, have been reported. Additionally, one pathogenic mutation, c.2969C>G (p.P990R), has been identified in bovine *NPC1* (Woolley et al., 2020; Woolley et al., 2021). To data, no such mutations have been identified in other animals, including dogs.

To date, very few cases of feline NPC have been reported (Somers et al., 2003; Zampieri et al., 2014; Mauler et al., 2017). In Japan, a Siamese cat and a Japanese domestic (JD) cat suspected of having NPC were described in 1989 (Yamagami et al., 1989; Kamiya et al., 1991; Kamiyu et al., 1991) and 1998 (Morozumi and Uchida, 1998), respectively. Recently, another Siamese cat was diagnosed with NP and proven to be affected by NP type A because the cat

was homozygous for a novel nonsense mutation (*SMPD1*:c.1017G>A (p.W339*)) (Takaichi et al., 2020). The first two cases were diagnosed as NP based on clinical manifestations and histopathological, ultrastructural, and tissue biochemical analyses, which were very similar to those of human and feline NPC cases (Yamagami et al., 1989; Kamiya et al., 1991; Kamiyu et al., 1991). Fortunately, some of the paraffin-embedded tissue specimens were stored for a long time and could be analyzed molecularly. This study aimed to identify feline-specific pathogenic mutation(s) in cats with NPC.

2.3. MATERIALS AND METHODS

This study was performed in accordance with the Guidelines Regulating Animal Use and Ethics of Kagoshima University (No. VM15041; approval date: 29 September 2015). Informed oral consent was also obtained from the owners of the cats with NPC (Yamagami et al., 1989; Morozumi and Uchida, 1998).

Specimen

For the DNA sequencing analysis, specimens were obtained from the paraffin-embedded liver and brain samples of two cats diagnosed with NP based on clinical, pathological, ultrastructural, and biochemical findings (Yamagami et al., 1989; Kamiya et al., 1991; Kamiyu et al., 1991; Morozumi and Uchida, 1998). Genomic DNA was extracted from the specimens using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and automated extraction equipment (magLEAD 6gC; Precision System Science, Co., Ltd., Matsudo, Japan) according to the manufacturer's recommendations.

Mutation Analysis

The coding exons and splice junctions of the feline *NPC1* and *NPC2* genes were amplified and assessed using polymerase chain reactions (PCR), and Sanger sequencing was done using specific primer pairs. The primer pairs were designed based on the reference sequences XM_019814307.3 (*NPC1*) and XM_003987833.6 (*NPC2*), as need arose because the DNA from the paraffin-embedded samples was modestly fragmented (sequences in Appendix-II). PCR was performed in a 20 μL reaction mixture containing 10 μL 2X PCR master mix (GoTaq Hot Start Green Master Mix, Promega Corp., Madison, WI, USA). The

PCR products were purified before sequencing using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Sanger sequencing was performed by Kazusa Genome Technologies Ltd. in Kisarazu, Japan. The obtained sequencing data were compared with the above-mentioned reference sequences to identify candidate pathogenic mutations. Multiple runs were performed in both directions for each cat to generate a consensus sequence. Sequence data were obtained from the two affected cats and four clinically healthy JD cats. Before analyzing *NPC1* and *NPC2* in the two cats, we confirmed the absence of the *SMPD1*:c.1017G>A (p.W339*) mutation, which causes NP type A (Takaichi et al., 2020).

Pathogenicity and stability prediction

Pathogenicity and the changes in protein stability upon mutations were analyzed using the PredictSNP and iStable servers, respectively. The PredictSNP server has an embedded algorithm for PredictSNP, MAPP, PhD-SNP, Polyphen-1, Polyphen-2, SIFT, and SNAP to classify variants as either "deleterious" or "neutral" (Bendl et al., 2014). Similarly, iStable has an embedded algorithm for iStable, i-Mutant, and MUpro to classify the variants as either "increasing" or "decreasing" the stability of the proteins (Chen et al., 2013; Chen et al., 2020; Sanavia et al., 2020). The i-Mutant 2.0 also gives the delta delta G (ddG) value, which is an indicator of the difference in free energy caused by the mutations. The amino acid sequences in the FASTA format and mutations were used as inputs for prediction. To compare and validate the pathogenicity of the identified candidate missense mutations, we also analyzed several pathogenic missense mutations that have already been reported in humans (*NPC1*:c.2974G>T, c.3019C>G, and c.3182T>C; *NPC2*:c.115G>A and c.358C>T) (Vanier et al., 2022), cats (*NPC1*:c.1322A>C and c.2864G>C) (Somers et al., 2003; Mauler et al., 2017), and cattle (*NPC1*:c.2969C>G) (Woolley et al., 2020; Woolley et al., 2021).

Preserving property

The preserving property of the amino acid sequence of the feline *NPC2* gene was analyzed and compared with that of several other animals using the Protein Basic Local Alignment Search Tool (Protein BLAST) provided by the National Center for Biotechnology Information (NCBI) site.

Pathogenicity prediction of the splice variant

The identified splice variant was analyzed for pathogenicity using IntSplice ver. 2.0 (Shibata et al., 2016). IntSplice predicts a splicing consequence of a single nucleotide variation at intronic positions -50 to -3 close to the 3'-end of an intron of the human genome. However, we were unable to perform predictions using the feline genome because of the lack of prediction algorithms. The position of the same nucleotide in the human genome was determined and predicted using a pathogenicity server, and a pathogenic probability score (> 0.5) was considered pathogenic (Shibata et al., 2016).

Preliminary survey

Preliminary survey was conducted on variants identified in this study using the 99 Lives Consortium dataset coordinated by the Lyons Feline Genetics Laboratory at University of Missouri.

2.4. RESULTS

Mutation detection

Sanger sequencing was performed on all the exons and splice junctions of the feline *NPC1* and *NPC2* genes in a Siamese cat and a JD cat diagnosed with NP. The sequenced exons and splice junctions were compared with the reference sequences XM_019814307.3 (*NPC1*) and XM_003987833.6 (*NPC2*). As a result, the sequencing and comparison in the feline *NPC1* gene revealed three homozygous silent mutations (c.54G>T (p.A18A), c.1815T>C (p.T605T), c.2214C>A (p.S605S)), one heterozygous silent mutation (c.2043C>T (p.P681P)), and one homozygous missense mutation (c.3224G>A (p.R1075H)) in the Siamese cat; nevertheless, no change was found in the JD cat. In the feline *NPC2* gene, one homozygous silent mutation (c.198A>C (p.S66S)) and one homozygous missense mutation (c.376G>A (p.V126M)) were identified in the Siamese cat (Figure 1). The JD cat was heterozygous for the same mutation (c.376G>A (p.V126M)) (Figure 1) and homozygous for a splice junction variant (c.364-4C>T (g.121865226C>T)) (Figure 2).

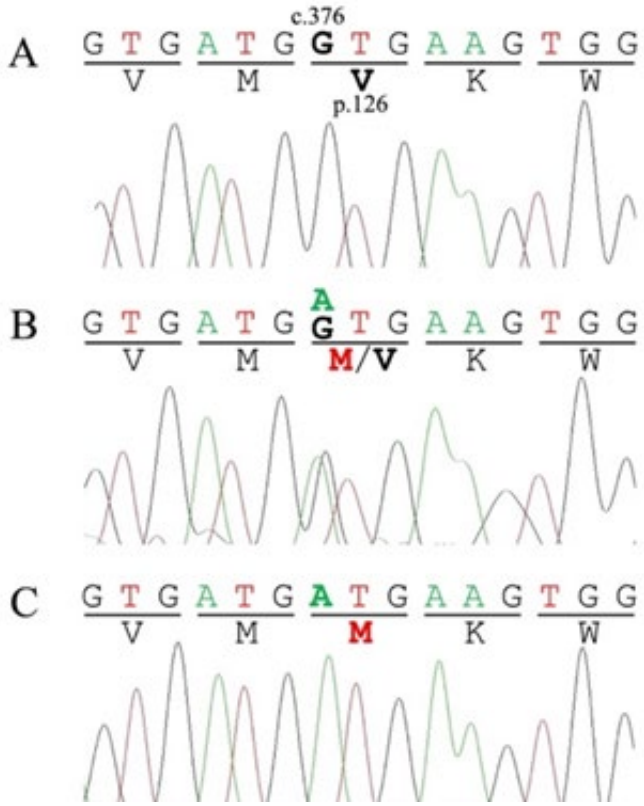

Figure 1. Partial genomic sequence electropherograms of exon 4 in the feline *NPC2* gene. Electropherograms from a clinically healthy control cat (A), a Japanese domestic cat (B) with Niemann-Pick disease type C (NPC), and a Siamese cat with NPC (C) are presented. The control cat is a homozygous wild-type (c.376G/G). The Japanese domestic and Siamese cats are heterozygous (c.376G/A) and homozygous (c.376A/A) for the c.376G>A (p.V126M) mutation, respectively.

Pathogenicity and stability prediction

The two missense mutations (*NPC1*:c.3224G>A (p.R1075H) and *NPC2*:c.376G>A (p.V126M)) identified in the two affected cats were further analyzed using the SIFT and PredictSNP servers, which included seven prediction algorithms (Table 1). In addition, we included five pathogenic mutations (three *NPC1* and two *NPC2* mutations) reported in humans, and two feline and one bovine *NPC1* mutations reported in animals as reference. The *NPC1*:c.3224G>A (p.R1075H) mutation was predicted to be neutral by six predictors, except

for SIFT which had a marginal score (0.05). In contrast, *NPC2*:c.376G>A (p.V126M) mutation was predicted to be pathogenic by all pathogenicity predictors, with a very low SIFT score (0.00). Other mutations already reported in humans and animals showed a deleterious effect on the catalytic site of the NPC1 or NPC2 protein in the analysis of all seven or at least five pathogenicity predictors.

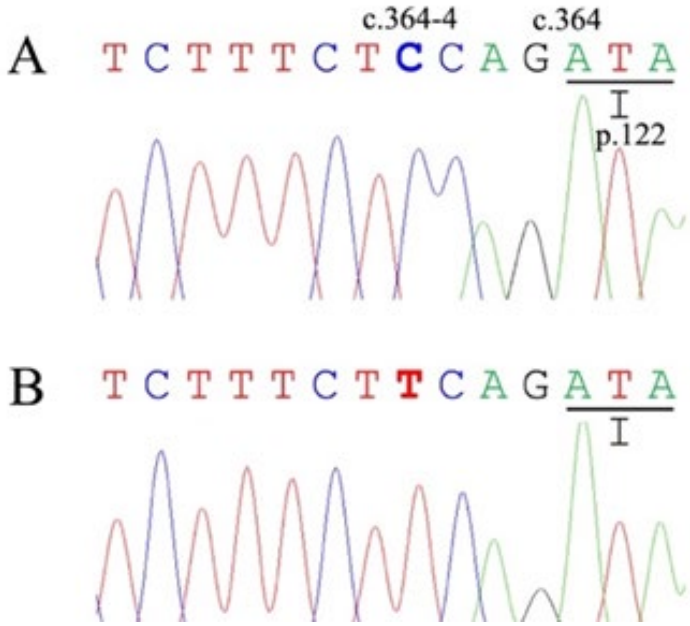

Figure 2. Partial genomic sequence electropherograms of the 5'-end of exon 4 in the feline *NPC2* gene. Electropherograms from a clinically healthy control cat (A) and a Japanese domestic cat (B) with Niemann-Pick disease type C (NPC) are presented. The control cat is a homozygous wild-type (c.364-4C/C). The Japanese domestic cat is homozygous (c.364-4T/T) for the c.364-4C>T variant.

Based on the results of all three stability predictors, analysis of the predicted stability using the iStable server showed decreased stability of the NPC2 protein produced by the *NPC2*:c.376G>A (p.V126M) mutation (Table 2). Regarding the *NPC1*:c.3224G>A (p.R1075H) mutation, two of three stability predictors showed decreased stability of the NPC1 protein produced by this mutation. Among the other mutations reported in humans and animals, three human mutations (NPC1:p.1007A and p.I1061T and NPC2:p.P120S) and a bovine

NPC1:c.2969C>G (p.P990R) mutation showed decreased stability in all three predictors. However, two human mutations (NPC1:p.G992W and NPC2:pV39M) were predicted to decrease the stability by one and two predictors, respectively. Among the reported feline *NPC1* mutations, the c.2846G>C (p.C955S) mutation was predicted to decrease stability based on two predictors. However, the feline *NPC1*:c.1322A>C (p.H441P) mutation was not predicted to decrease the stability by any predictor, but two predictors predicted an increase in the stability.

In association with the feline *NPC2*:c.376G>A (p.V126M) mutation, the preserving property of an amino acid residue of p.V126 was surveyed in a variety of animals using Protein BLAST (Figure 3). As a result, valine at position 126 was found to be highly preserved in many mammals including dogs, cattle, sheep, horses, dolphins, humans, macaques, mice, and tigers.

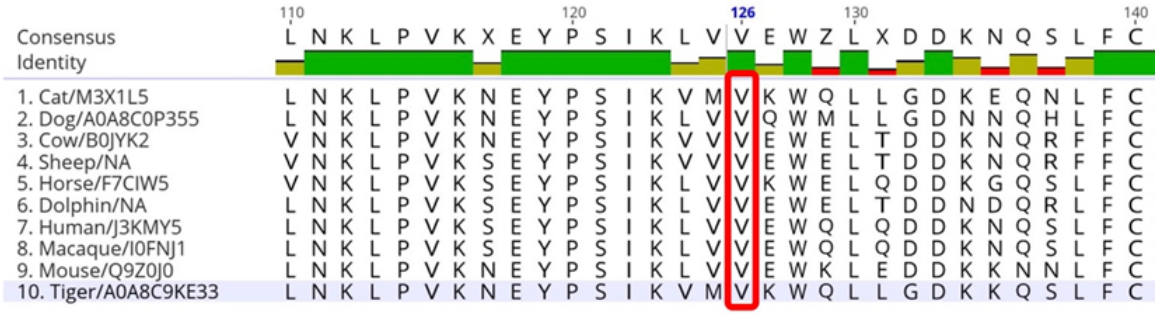

Figure 3. Multiple amino acid sequence alignment at position p.126V in the *NPC2* gene of different mammals. Valine at position 126 is highly preserved in these mammals.

Splice variant analysis

A homozygous variant in the splice region (*NPC2*:c.364-4C>T), identified in a JD cat with NP, was analyzed using IntSplice ver. 2.0. This variant was predicted to be normal with a low pathogenic probability score (0.106938).

Preliminary survey

The preliminary survey was conducted on two missense variants (*NPC1*:c.3224G>A (p.R1075H) and *NPC2*:c.376G>A (p.V126M)) and a splice variant (*NPC2*:c.364-4C>T), using the 99 Lives Consortium dataset that currently consists of whole genome sequence data from 362 domestic cats and whole exome sequence data from 62 cats. The survey result showed that these three variants were absent in the database.

Table 1. Analysis of missense mutations in the catalytic site of the NPC1 and NPC2 proteins using the SIFT and PredictSNP servers.

Gene	Species	Mutation		SIFT		Predict SNP	MAPP	PHD-SNP	Polyphen-1	Polyphen-2	SNAP
		Nucleotide	Amino acid	Score	Interpretation						
	Cat*	c.3224G>A	p.R1075H	0.05	N/D	N	N	N	N	N	N
	Cat	c.1322A>C	p.H441P	0.25	N	D	D	D	D	D	D
	Cat	c.2864G>C	p.C955S	0.00	D	D	D	D	D	D	N
NPC1	Cattle	c.2969C>G	p.P990R	0.00	D	D	D	D	D	D	D
	Human	c.2974G>T	p.G992W	0.03	D	D	N	D	D	D	N
	Human	c.3019C>G	p.P1007A	0.00	D	D	D	D	N	D	D
	Human	c.3182T>C	p.I1061T	0.02	D	D	D	D	D	D	N
	Cat**	c.376G>A	p.V126M	0.00	D	D	D	D	D	D	D
NPC2	Human	c.115G>A	p.V39M	0.01	D	D	D	D	D	D	D
	Human	c.358C>T	p.P120S	0.00	D	D	D	D	D	N	D

D: deleterious; N: neutral; UK: unknown.

Table 2. Prediction of stability of NPC1 and NPC2 proteins for selected missense mutations by protein stability predictors.

Gene	Species	Mutation		i-Mutant 2.0		MUpro		iStable	
		Nucleotide	Amino acid	Result	ddG	Result	Conf. score	Result	Conf. score
NPC1	Cat*	c.3224G>A	p.R1075H	Decrease	-1.83	Null	Null	Decrease	0.78063
	Cat	c.1322A>C	p.H441P	Increase	0.35	Null	Null	Increase	0.631442
	Cat	c.2864G>C	p.C955S	Decrease	-0.59	Null	Null	Decrease	0.556555
	Cattle	c.2969C>G	p.P990R	Decrease	-0.47	Decrease	-0.093720996	Decrease	0.848305
	Human	c.2974G>T	p.G992W	Increase	0.10	Decrease	-0.13709342	Increase	0.573621
	Human	c.3019C>G	p.P1007A	Decrease	-1.16	Decrease	-1	Decrease	0.681309
	Human	c.3182T>C	p.I1061T	Decrease	-2.28	Decrease	-1	Decrease	0.826884
NPC2	Cat**	c.376G>A	p.V126M	Decrease	-1.03	Decrease	-0.96085544	Decrease	0.799741
	Human	c.115G>A	p.V39M	Null	-0.69	Decrease	-0.63162291	Decrease	0.620853
	Human	c.358C>T	p.P120S	Decrease	-1.83	Decrease	-0.33095521	Decrease	0.887135

2.5. DISCUSSION

Clinical manifestations, serum biochemistry, and imaging tests such as magnetic resonance imaging are common tools for the premortem diagnosis of lysosomal storage diseases, except when the molecular causes are known (Vite et al., 2008). These clinical diagnostic tools are not enough to differentially diagnose metabolic diseases from other related diseases with similar clinical manifestations. Necropsy findings and further pathological and laboratory investigations allow us to approach the diagnosis, but it is still difficult to diagnose genetic diseases without any molecular diagnostic tools, such as PCR, especially in the veterinary field. This is because of insufficient information about the molecular bases of genetic diseases in animals. Thus, once a causative mutation is identified in an animal, breed, or family, simple procedures can be used for rapid genetic diagnosis and genotype screening, leading to effective disease control and the eradication of the disease (Mizukami et al., 2011; Pervin et al., 2022). For this purpose, stored paraffin-embedded specimens of animals suspected of having genetic diseases can be utilized if a tentative diagnosis has already been established. We recently used DNA extracted from a paraffin-embedded specimen of a cat with a genetic disease, and successfully identified the first pathogenic mutation in feline Pompe disease, which is a glycogen storage disease type II (Rakib et al., 2023).

Two cats suspected to have NPC have been described in Japan in 1989 (Yamagami et al., 1989; Kamiya et al., 1991; Kamiyu et al., 1991) and 1998 (Morozumi and Uchida, 1998). The first case involved a 6-month-old female Siamese cat that showed neurological signs such as an ataxic gait, head tremors, loss of equilibrium, and hepatomegaly (Yamagami et al., 1989). This cat died at 11 months of age due to the progression of neurological dysfunction, and was diagnosed with neurovisceral sphingomyelinosis postmortem, with an accumulation of other types of lipids based on lipid analysis, histopathology, electron microscopy, and lectin histochemistry (Yamagami et al.,

1989; Kamiya et al., 1991; Kamiyu et al., 1991). The second case was a 3-month-old male JD cat with progressive neurological signs such as ataxia, generalized tremors, and impaired vision. The JD cat showed increased liver enzyme activity in serum biochemistry and had brain atrophy, as suggested by computed tomography. The cat died at 5 months of age due to the progression of neurological dysfunction. Postmortem, the disease proved to be neurovisceral NP with an accumulation of sphingomyelin and cholesterol based on lipid analysis, histopathology, and electron microscopy (Morozumi and Uchida, 1998). The clinical, biochemical, and histopathological characteristics of these two cats with NP were similar to those of NPC rather than those of NP type A. Consequently, we analyzed the feline *NPC1* and *NPC2* genes after confirming the absence of the *SMPD1*:c.1017G>A (p.W339*) mutation that was recently reported in a Siamese cat with NP type A (Takaichi et al., 2020).

Sanger sequencing of the feline *NPC1* and *NPC2* genes of the two cats with NP, and comparison with the reference sequences were performed to identify candidate pathogenic mutations in feline NPC. As the candidates of potential pathogenic mutations, two missense mutations (*NPC1*:c.3224G>A (p.R1075H) and *NPC2*:c.376G>A (p.V126M)) and one splice variant (*NPC2*:c.364-4C>T (g.121865226C>T)) were found in the Siamese and/or JD cats (Figures 1 and 2). After the evaluation of pathogenicity and stability prediction (Tables 1 and 2) with the comparison of the reference data obtained from several known pathogenic mutations in humans and animals, the *NPC2*:c.376G>A (p.V126M) mutation was selected as the most likely candidate pathogenic mutation in these cats with NPC. This is because this mutation was evaluated to be deleterious by all seven pathogenicity predictors, and was observed to decrease the stability of all three predictors. In contrast, the *NPC1*:c.3224G>A (p.R1075H) mutation was ruled out as a pathogenic mutation based on the evaluation data (mostly neutral) obtained from the pathogenicity

predictors (Table 1), although two of the three stability predictors showed decreased stability (Table 2). Furthermore, the *NPC2*:c.364-4C>T (g.121865226C>T) splice variant identified in the JD cat was suspected to be benign based on the splice variant analysis data; however, further studies are required to determine the relationship between this variant and NPC.

The preliminary survey using the 99 Lives Consortium dataset indicated that *NPC1*:c.3224G>A, *NPC2*:c.376G>A, and *NPC2*:c.364-4C>T were absent, suggesting that the three variants may be rare in the domestic cat population and can be causal for feline NPC. However, the 99 Lives Consortium dataset does not include the data from many Asian cats; therefore, a large-scale survey using Asian cat population would be beneficial in demonstrating the exact frequencies of these variants.

The Siamese cat was homozygous for the *NPC2*:c.376G>A (p.V126M) mutation, whereas the JD cat was heterozygous for the same mutation. Therefore, the JD cat may be a compound heterozygote with two different mutations. A counterpart pathogenic mutation of *NPC2* may be located in the intronic regions that could not be analyzed in this study. When comparing the clinical manifestations of the two NPC-affected cats, the disease onset in the JD cat was earlier (at three months old) than that in the Siamese cat (at six months old) (Yamagami et al., 1989; Morozumi and Uchida, 1998). Furthermore, the JD cat died at an earlier age (five months old) than the Siamese cat (11 months). This suggests that the disease progression is faster in the JD cat than in the Siamese cat. Therefore, another mutation in the JD cat may be as deleterious as a null mutation, such as a splice defect. In the first report of two littermate cats with NPC2 disease caused by a splice defect (*NPC2*:c.82+5G>A (p.G28_S29ins35)), the disease onset was approximately three months old, and one cat died at 10 months of age, but the other littermate was alive for more than one year until euthanasia (Zampieri et al., 2014). The clinical severity of these littermates with

NPC2 disease was similar to that of our JD cat but not to that of the Siamese cat in this study. This also suggests that the JD cat may have a null mutation as a counterpart mutation of *NPC2*:c.376G>A (p.V126M).

Treatment efforts for human NPC are currently focused on slowing disease progression (Sitarska et al., 2021). The only registered drug that is promising for the treatment of patients with NPC is N-butyldeoxynojirimycin (miglustat), which was initially approved for the treatment of Gaucher disease, a hereditary deficiency of lysosomal glucocerebrosidase. Miglustat is an iminosugar that inhibits glucosylceramide synthase, which is required in the early stages of glycosphingolipid synthesis. The oral administration of miglustat (1,200 mg/kg/day) in mice with NPC (BALBc/NPCnih) reduced the accumulation of gangliosides in the brain, slowed the neurological progression, and increased the lifespan by approximately 33% (Zervas et al., 2001). Subsequent studies on cats with NPC caused by *NPC1*:c.2864G>C (p.C955S) showed that the oral administration of miglustat (50 mg/kg/day) was effective in reducing the accumulation of gangliosides in the brain, delaying the onset of neurological symptoms, and increasing the lifespan by approximately 74% (Zervas et al., 2001). Other effective treatments are still under development, and combination therapies using several compounds and other strategies, such as gene therapy, are increasingly being worked on (Sitarska et al., 2021).

A colony of cats with NPC caused by *NPC1*:c.2864G>C (p.C955S) is kept at Colorado State University (Zervas et al., 2001; Sitarska et al., 2021). As described above, cats are being utilized as a large animal model for NPC studies, including the development of new therapeutic methods (Zervas et al., 2001). However, to the best of our knowledge, a cat colony with *NPC2*-derived disease has not yet been established. Notably, there are differences between *NPC1*- and *NPC2*-derived diseases that require both animal models for an accurate study. We suppose that cats

carrying the *NPC2*:c.376G>A (p.V126M) mutation are present in the cat population in Japan, which could be instrumental in future therapeutic research as a large animal model for human NPC2 disease.

CONCLUSION

Based on the results obtained, chapter 1 identified a pathogenic mutation (*GAA*:c.1799G>A, p.R600H) in the feline *GAA* gene of a DSH cat with PD. This is the first report of a cat with PD carrying the same mutation as reported in a case of human classical IOPD. The clinical and histological findings in this cat with PD were similar to those in humans with IOPD. Therefore, this feline PD is an excellent model of human PD, especially IOPD. This feline model of PD may contribute to the development of new therapeutic strategies for treating human PD.

Furthermore, we identified a pathogenic mutation (*NPC2*:c.376G>A (p.V126M)) in the feline *NPC2* gene of a Siamese cat and a JD cat with NP. The pathogenicity of this mutation was confirmed based on the data obtained from a variety of predictors by comparing data from several known pathogenic mutations. The Siamese and JD cats were homozygous and heterozygous for this mutation, respectively. No other exonic *NPC2* mutation or deleterious splice variant was found in the JD cat, suggesting that this cat was a compound heterozygote of the identified *NPC2*:c.376G>A mutation and another pathogenic mutation that may be located in intronic regions.

Finally, this study identified a pathogenic mutation in the feline *GAA* gene, making it an excellent model for human Pompe disease. Additionally, a second pathogenic mutation in the feline *NPC2* gene was discovered, providing insights into Niemann-Pick disease in both feline and human populations.

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Appendix-I

Table I-1: Primers used to amplify exons and splicing regions of *GAA* gene

Exon no.	Name	Primer sequence (5′→3′)	Length (mer)	GC (%)	T_m (ºC)	Amplicon (bp)	T_a (ºC)
1	UTR	Not sequenced					
2	fGAA-E2a-F	ATTGGCGTGAGTCCCCTGAG	20	60	55.9	344	57
	fGAA-E2a-R	AGCTGCTCTGGGACTGCCGT	20	65	57.9		
	fGAA-E2a-F2	CGGCCCTTCTCTCAGCA	17	65	57	222	58
	fGAA-E2a-R2	TCGATAAAGCCCCTCAGCT	19	50	57		
	fGAA-E2b-F	GGGCTTTATCGAGCTCGCCA	20	60	55.9	364	58
	fGAA-E2b-R	GCACGTCCAGGCGTAAGGTCA	21	62	58.3		
	fGAA-E2b-F2	GCTCCATGATTTCTTGCTG	19	45	55	219	56
	fGAA-E2b-R2	ACACTGCTCCTGGGTGA	17	58	55		
	fGAA-E2c-F	AGCTGCACAACCTGACCACCA	21	57	56.3	251	57
	fGAA-E2c-R	GACTCCCCGTGAAGGCACAA	20	60	55.9		
	fGAA-E2c-F2	CCCAACAGCCGCTTCGAC	18	63	60.8	249	59
	fGAA-E2c-R2	GGCGTAAGGTCAAGATGTCC	20	55	60.5		
3	fGAA-E3-F	CTCCTGGAGGGTGAGGAAG	20	63	55.4	328	56
	fGAA-E3-R	CAGGATCGCTGTGTATGGTG	20	55	53.8		
	fGAA-E3a-F2	CTCCTGGAGGGTGAGGAA	18	63	58.4	183	58

	fGAA-E3a-R2	CTGGAGGTCCACGCTGTA	28	55	58.4		
	fGAA-E3b-F2	CTCATCCACACTTTACAGCG	20	55	58.4	175	58
	fGAA-E3b-R2	ATCGCTGTGTATGGTGGAGT	20	55	58.4		
4	fGAA-E4-F	CGTCAGTGCATCACCGTGGTG	21	62	58.3	348	58
	fGAA-E4-R	CGTTAGCAGCCTCACCCACATG	22	59	58.6		
5	fGAA-E5-F	GTACAAGGGGCAGTGAAGACG	21	57	56.3	257	57
	fGAA-E5-R	CTGGCCTGGGAGCTCAGAAG	20	65	57.9		
6	fGAA-E6-F	AGCAGGCTGTGGATTGGTGGC	21	62	58.3	343	58
	fGAA-E6-R	CGGCATGAATGGGGAGCCTG	20	65	57.9		
	fGAA-E6-F2	CAGTATGGGGCGCAGGGAT	18	61	60.8	282	60
	fGAA-E6-R2	TCTGTGAGCGCGTGGGCA	18	61	60.8		
7	fGAA-E7-F	CTGGAAGTCGTGGGTAGGCTG	21	62	58.3	345	58
	fGAA-E7-R	ACTGCGTGTCCTGTGGCCACA	21	62	58.3		
	fGAA-E7-F2	TGCCCACGCGCTCACAGA	18	61	60.8	221	60
	fGAA-E7-R2	CAGCACAGGGACGGAGTC	18	61	60.8		
8	fGAA-E8-F	GCCAGGTGGTGGAGAACATGA	21	57	56.3	301	57
	fGAA-E8-R	TCCCTTCCCTAACAGCTGGCA	21	57	56.3		
9	fGAA-E9-F	TCTGTCCTCCGTCAACGCTG	20	60	55.9	323	56
	fGAA-E9-R	CCTGTCCTCTTCCTGAACGC	20	60	55.9		
	fGAA-E9-F2	AGTGGGTGCCTGGCTTTG	18	61	58.4	229	58
	fGAA-E9-R2	CCGTTTTCTGCCCTCTCC	18	61	58.4		
10,11	fGAA-E10,11-F	CAGGCAGGTGAGCGAGGCT	19	68	57.6	447	56

	fGAA-E10,11-R	CTGAGTCTCCCAAACCAGAG	20	55	53.8		
	fGAA-E10-F2	GGCAGGTGAGCGAGGCT	17	71	59.8	249	58
	fGAA-E10-R2	AGACAGGCCCCGGAGGT	17	71	59.8		
	fGAA-E11-F2	AAGTGTCCCGTGGGCTGT	18	61	58.4	241	58
	fGAA-E10-R2	AGCCCTGATCTGAGCCTC	18	61	58.4		
12	fGAA-E12-F	CAGAGGAGACGATACAGGGAC	21	57	56.3	301	58
	fGAA-E12-R	TCTGGGAGTCTGTGTGCGGTC	20	62	58.3		
13	fGAA-E13-F	CGGCTCATCATCCAGGGTGGT	21	62	58.3	381	58
	fGAA-E13-R	ATGGCAGCGGCATCTCCCTGA	21	62	58.3		
	fGAA-E13-F2	TTGCTCTCGGCCCTGCCA	18	67	60.8	222	58
	fGAA-E13-R2	TGACAGGCGCTCTCACCT	18	67	58.4		
14	fGAA-E14-F	TGGCTACAACGTCTGCCGGG	20	64	57.9	441	58
	fGAA-E14-R	AACCTGTACGGCTCCTGAGGC	21	62	58.3		
	fGAA-E14-F2	GCCCAGGTGGACCTACTC	18	67	60.8	286	60
	fGAA-E14-R2	TCCGCTCTCACTGCCGGT	18	67	60.8		
15	fGAA-E15-F	AACGACCTCCACAGCCTGGTAG	22	62	58.6	454	58
	fGAA-E15-R	CCTGAGCTGCACTCTCATGGG	21	62	58.3		
	fGAA-E15-F2	TGAGAGCGGAGCCGGTCT	18	67	60.8	280	59
	fGAA-E15-R2	TGTGGCAGGACGGGGCT	17	71	59.8		
16	fGAA-E16-F	AGTTTGCTGGAAAGGCCGGCAG	22	59	58.6	491	58
	fGAA-E16-R	TCCTGGGGAGCTACCCACTTG	21	62	58.3		
17	fGAA-E17-F	CCTCTGTCCCAACGACTTGT	20	55	53.8	459	56

	fGAA-E17-R	CCTCACACGCTGTCGTCTCT	20	60	55.9	279	59
	fGAA-E17-F2	CTGTCCCAACGACTTGTCTG	20	55	60.5		
	fGAA-E17-R2	ACGGACCCTCTACTGGACA	19	58	59.5		
18	fGAA-E18-F	TCCTCCAGGGTGTCTGCCTC	20	65	57.9	427	58
	fGAA-E18-R	CCTGCTCGCTTCACTTGGCTG	21	62	58.3		
19	fGAA-E19-F	TGACCGCGGTCCCCATGCA	19	68	57.6	343	58
	fGAA-E19-R	GTCCCCGGGGTCCTTGGTA	19	68	57.6		
20	fGAA-E20-F	AACAGAGCCGGAAGCCTCCAG	21	62	58.3	326	58
	fGAA-E20-R	AACTGCTTCCGACCCTGAGCC	21	62	58.3		

T_m: Melting temperature (salt adjusted), T_a: Annealing temperature used

Appendix-II

Table II-1. Primers used to amplify exons and splicing regions of the feline *NPC1* gene

Exon no.	Name	Primer sequence (5′→3′)	Length (mer)	GC (%)	T_m (ºC)	Amplicon (bp)	T_a (ºC)
1	fNPC1-E1-F	AACCGCACCAAGGCAGCATG	20	60	57.7	133	56
	fNPC1-E1-R	TAGCCTCCCGGCCCTCCTC	19	74	57.9		
	fNPC1-E1-F1	GCCTAGTCAAGTCGAGTCTCG	21	57	56.3		
2	fNPC1-E2-F	GGCCCCGATGAAACTGAG	18	60	58.4	193	59
	fNPC1-E2-R	GTTCCCAGCGCCCAAGATAAC	21	57	63.2		
3	fNPC1-E3-F	GAGTAATGTGTGTCCTGATTTGAC	24	42	62	162	60
	fNPC1-F3-R	AACTGTAGAGGCAGTGCAAGG	21	52	61.2		
	fNPC1-E3-F2	CTCTGTCCAGGATTCTTCTTTG	22	45	60.1	92	59
	fNPC1-E3-R2	TGTAGAGGCAGCTGCAGGT	19	58	59.5		
	fNPC1-E3-F3	GAGTAATGTGTGTCCTGATTTG	22	41	58.4	175	55
	fNPC1-E3-R3	ACTGTAGAGGCAGTGCA	17	53	52.4		
4	fNPC1-E4-F1	GCTGGCCCTATTATCTGTGA	20	50	58.4	165	58
	fNPC1-E4-R1	GTAACAGGATCAACATAATCTTCAG	25	36	60.9		
	fNPC1-E4-F2	GGCAAAGTCAGTTTCTGAATG	21	43	57.5	166	57
	fNPC1-E4-R2	TAAAAGCTTATTCACCCTCACC	22	41	58.4		

5	fNPC1-E5-F	TTGCCTTCTTCCTCTGCCAG	20	55	60	133	60
	fNPC1-E5-R1	GTACTCAATCCAGTTAGTGGCA	22	55	60		
	fNPC1-E5-F1	ATGACAAAGCCCTGGGACTG	20	55	60	150	58
	fNPC1-F5-R2	AATACACCAAACTTGGGAGTC	21	43	57.5		
6	fNPC1-E6-F	ATTTACTCAACCCCATCCTCCC	22	50	62	185	59
	fNPC1-E6-R	TGCTCACCTGTAGCACCAC	19	58	59.5		
	fNPC1-E6-F1	TCAATTGTTTGTGGCCCAAAGC	22	45	60	183	60
	fNPC1-E6-R1	CAAGATTCTCCAGGGCACAG	20	55	60		
	fNPC1-E6-F2	TGTCAGTGTGCTTAGCACTAG	21	48	59.5	282	57
	fNPC1-E6-R2	CTTGCCCTGGGGAATAAAC	19	53	57.5		
7	fNPC1-E7-F	GGTCTATGGGACTATCAGAGGAAC	24	50	65	168	60
	fNPC1-E7-R	CAAAAGTGCCTACCTCTGTCATTG	24	46	60		
8	fNPC1-E8-F1	GACTTTCAGGAATGGCTTGGC	21	50	61	152	59
	fNPC1-E8-R1	GTTTCGGACGCAGAAAGAGC	20	55	60		
	fNPC1-E8-F2	GCATTTGAGGGCTGTCTGAG	20	55	60	124	60
	fNPC1-E8-R2	CAAACACCAGGCCTGAAGAAC	21	52	61		
	fNPC1-E8-F3	GTGTTTGTGCGGGTCACGA	19	58	60	131	59
	fNPC1-E8-R3	GCCTGGATGATGAGCTGCT	19	58	60		
	fNPC1-E8-F4	GCACTTTGGGCCTTTCTTCC	20	55	60	144	59
	fNPC1-E8-R4	ACGTTACCTGGTGCAAGATC	20	50	58		
	fNPC1-E8-F5	TTCTTCTCCCTGGCCTTCATTG	22	50	62.1	173	60

	fNPC1-E8-R5	GGCTTCCCAGAACGGATTTC	20	55	60.5		
	fNPC1-E8-F6	GTAGCTAACGCTTTCCCTGT	20	50	58.4		
9	fNPC1-E9-F1	ATCCAATGTGTTTCTGGGTTTG	22	41	58	131	58
	fNPC1-E9-R1	GGGCCACGCAGATGTCTTG	19	63	62		
	fNPC1-E9-F2	CTGCCATCGAAAATATCACTGC	22	45	60	142	60
	fNPC1-E9-R2	AGCATGGAATGGCTGTTCTG	22	45	60		
	fNPC1-E9-F3	CTGCACCATTCTGAGCGTG	19	58	60	157	58
	fNPC1-E9-R3	CTGGTGGGCCGAATGATG	18	61	58		
10	fNPC1-E10-F	GCTTGTTGTAACTGGGAGTTG	21	48	60	99	59
	fNPC1-E10-R	CACCTGTTAGTGACAGAACCA	21	48	60		
	fNPC1-E10-F1	CGATACCAGTTTGCTCCATG	20	50	58.4	149	58
	fNPC1-E10-R1	ACCGAATGTACCCAGGCAAG	20	55	60.5		
11	fNPC1-E11-F1	CAATAATGCCACAGCCCTTG	20	50	58.4	117	58
	fNPC1-E11-R	CACACAACAAAGACAGCAACTC	22	45	60		
	fNPC1-E11-F2	CTGCACACTTCTTATCTTCT	20	40	54.3	124	57
	fNPC1-E11-R1	CTGGAGCCTCTCTGTATCAT	20	50	58.4		
12	fNPC1-E12-F1	GTCTCACTTAGCTGTCACTAAG	22	45	58	159	57
	fNPC1-E12-R1	CACCGTTACTTTCACGATTTAG	22	41	58		
	fNPC1-E12-F2	ACTGAGCGAAGTATTGAAGATG	22	41	58	167	58
	fNPC1-E12-R2	CCAGCACAGACACACACACA	20	55	60		
13	fNPC1-E13-F1	GGACAAATGATACGCTGACAG	21	48	59.5	155	58

	fNPC1-E13-R1	CAATGAGGGTGAGGGGGAT	20	50	59.5		
	fNPC1-E13-F2	TGCTCGTTGGGCATCTTTAG	19	58	59.5	162	58
	fNPC1-E13-R2	TGGTCAGATGCCCAGCTCT	19	58	59.5		
14	fNPC1-E14-F1	GAAGCAAAGAGATGGCATCG	20	50	58.4	131	57
	fNPC1-E14-R1	CACTTCTCCTAGGACCCT	18	56	56.3		
	fNPC1-E14-F2	AACTCTGGATCAGCAGCTG	19	53	57.5	137	56
	fNPC1-E14-R2	CCAGGCTTAGAAAACTGTAGG	21	48	59.5		
	fNPC1-E14-F3	CGAGATGAACGTCTTCATGG	20	50	58.4		
15	fNPC1-E15-F1	CAGAGTCCTGTGCTGCAG	18	61	58.4	127	58
	fNPC1-E15-R1	AGAAGGAAGTCGATGAGGAC	20	55	58.4		
	fNPC1-E15-F2	CTCTCTGTTTGCCGGGAT	18	56	56.3	130	56
	fNPC1-E15-R2	GACTCTAATTAGGAGACTGTG	21	43	57.5		
16	fNPC1-E16-F	GTCTAGAATCTGACTGAAGCAG	22	45	60.1	144	59
	fNPC1-E16-R1	CCGAAACAAGCAGCTCTCTG	22	45	60.5		
	fNPC1-E16-F1	GAAGATGGAACCAGTGTCCAG	21	54.4	61.2	155	60
	fNPC1-E16-R	CTTAGAATGCATGGGACAGATC	22	45	60.1		
17	fNPC1-E17-F	GCAATCACTGTCTTATAACTCAC	23	39	59.2	115	58
	fNPC1-E17-R1	GAAAGAGACTGATCCAATCCG	21	48	59.5		
	fNPC1-E17-F1	GTCATTCAGTGTTGCAGTCC	20	50	58.4	114	58
	fNPC1-E17-R	TTTGTTCACACCACTGGCTCA	21	48	59.5		
18	fNPC1-E18-F1	CGCCTTGCTTAGTTACTATCAG	22	45	61.1	187	58

	fNPC1-E18-R1	CCGCACACCATGTTCTGC	18	45	60.1		
	fNPC1-E18-F2	TTTGTCCTGGAGGAAGGGCA	20	55	60.5	157	60
	fNPC1-E18-R2	GCTTCTGAAGTACAAGACAAGG	22	45	60.1		
	fNPC1-E18-F3	GTTCACACTCTCTCCTATTCTT	22	41	60.5	103	59
	fNPC1-E18-R3	CCTTTCAGAGAGGTGTAGTC	20	50	58.4		
	fNPC1-E18-F4	ACTCCTACGTGATGGATTATTTC	23	39	59.2		
19	fNPC1-E19-F	AGACTTCCTGCCTGTGGAG	19	58	59.5	130	58
	fNPC1-E19-R1	CTACAGCAAGAAGACTGAGG	20	50	58.4		
	fNPC1-E19-F1	CGACGATTACTTTGATTGGGTC	22	45	60.1	133	59
	fNPC1-E19-R	CAAAAGTCTTCAGAGAAAAGGG	22	41	58.4		
20	fNPC1-E20-F	CTGGAGACTAATCTTCTGACC	21	48	58.3	143	58
	fNPC1-E20-R1	GCAAACAGAGGCCTCAAGGT	21	48	59.5		
	fNPC1-E20-F1	GCAAACAGAGGCCTCAAGGT	20	55	60.5	123	59
	fNPC1-E20-R	GAGGCAGGCACGTATCTGA	19	58	59.5		
21	fNPC1-E21-F1	TCTGCAGATCCTGTTCTGAC	20	50	58.4	163	58
	fNPC1-E21-R1	CTCATGGCGTCAGTAAAGTC	20	50	58.4		
	fNPC1-E21-F2	CACACCGTGCTTCAGACGT	19	58	59.5	171	58
	fNPC1-E21-R2	GGGTGTGGGCTCTAACCTA	19	58	59.5		
22	fNPC1-E22-F1	GCGAGAATGAGCTGTGACAG	20	55	60.5	178	59
	fNPC1-E22-R1	CATGATCACTGCAGACCACA	20	50	58.4		
	fNPC1-E22-F2	CGTGATTCTCCTGGGCTGT	20	50	59.5	177	58

	fNPC1-E22-R2	GGACACAGGAGTTATGCACTG	21	52	61.2		
23	fNPC1-E23-F	AAGGATGTAACGGAAGCACTT	21	43	57.5	188	56
	fNPC1-E23-R	ACGAGCTGGGCACTTTATG	19	53	57.5		
24	fNPC1-E24-F1	GAGTAGGTTGGTAAGTGCTTTC	22	45	60.1	164	57
	fNPC1-E24-R1	TGGGTGGCTCCCAGTAAGA	19	58	59.5		
	fNPC1-E24-F2	CTTCAGGATGTATTTGGCTATG	22	41	58.4	141	58
	fNPC1-E24-R2	GATCTTTCTTCTCCTTTAGTAGC	23	39	59.2		
25	fNPC1-E25-F	GAACTACCCTGTCGCCTTC	19	58	59.5	180	58
	fNPC1-E25-R	TTCAGAGTCCCCCGACCT	18	61	58.4		

T_m: Melting temperature (salt adjusted), T_a: Annealing temperature used.

Table II-2. Primers used to amplify exons and splicing regions of the feline *NPC2* gene

Exon no.	Name	Primer sequence (5′→3′)	Length (mer)	GC (%)	T_m (ºC)	Amplicon (bp)	T_a (ºC)
1	fNPC2-E1-F	AGGCGGTCGCTTCTTCCT	18	61	58.4	141	58
	fNPC2-E1_R	GCTCCGGCTCACCGCA	16	75	58.4		
	fNPC2-E1-F2	TCTTGTGACTGAGGCGGTC	19	58	59.5		
2	fNPC2-E2-F	CAGGTGGTTTATCTTGCCTC	20	50	58.4	192	58
	fNPC2-E2-R	TGAATTTGAAGTCAAGAGCCAC	22	41	58.4		
3	fNPC2-E3a-F	GCTGGCCCTGTCACTATC	18	61	58.4	137	58

	fNPC2-E3a-R	CACTCTTACAACCATCAGCC	20	50	58.4		
	fNPC2-E3a-F2	CTGTCACTATCTCATTCTCTTTC	23	39	59.2		
	fNPC2-E3b-F	GATGGGCGTAGCAGTTCC	18	61	58.4	170	59
	fNPC2-E3b-R	TCACAAGGCTACCAGTGTC	19	53	57.5		
	fNPC2-E3b-F2	GGCATCCTGATGGGCGT	17	61	57.3	133	56
	fNPC2-E3b-R2	ACCAGTGTCCTGAACAACG	19	53	57.5		
	fNPC2-E3b-F3	GCTTTGGTGTATGGCATCCT	20	50	58.4		
4	fNPC2-E4-F	ACCGGTTCTGGATTGAGGT	19	53	57.5	158	58
	fNPC2-E4-R	AGCACCTCTAGCCTTCAATC	20	50	58.4		
	fNPC2-E4-F1	GTCTGAGGAGTTAGGCAGTT	20	50	58.4		

T_m: Melting temperature (salt adjusted), T_a: Annealing temperature used

Table II-3. Primers used to amplify exons and splicing regions of the feline *SMPD1* gene

Exon no.	Name	Primer sequence (5′→3′)	Length (mer)	GC (%)	T_m (ºC)	Amplicon (bp)	T_a (ºC)
2	fSMPD1-E2-F	GAGCACACCTGTCAATGGCT	20	55	60.5	148	58
	fSMPD1-E2-R	GGTTTCCATGGACCTCAACTA	21	48	59.5		

T_m: Melting temperature (salt adjusted), T_a: Annealing temperature used.