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RESEARCH REPORT

Dihydropyrimidinase Deficiency: The First Feline Case of Dihydropyrimidinuria with Clinical and Molecular Findings

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Abstract Dihydropyrimidinase (DHP, EC 3.5.2.2) is the second enzyme of the pyrimidine degradation pathway and a deficiency of this enzyme is responsible for a rare inborn metabolic syndrome characterized by dihydropyrimidinuria. Here we report a cat with DHP deficiency, manifesting malnutrition, depression, vomiting, and hyperammonemia. A gas chromatographic–mass spectrometric analysis of urinary metabolic substances showed the presence of large amounts of dihydrouracil and dihydrothymine and moderate amounts of uracil and thymine, suggesting DHP deficiency. Analysis of the feline *DPYS* gene encoding DHP demonstrated that the cat was homozygous for the missense mutation c.1303G>A (p.G435R) in exon 8, which corresponds to a known mutation in a human patient with DHP deficiency. Population screening in 1,000 cats did not reveal any animal possessing this mutation, suggesting the prevalence of the mutant allele to be very low. This is the first report of naturally occurring DHP deficiency in animals and the cat represents a model of the human disease.

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Introduction

Dihydropyrimidinase (DHP, EC 3.5.2.2) is the second enzyme involved in the breakdown of the pyrimidine bases, that is, uracil and thymine, and catalyzes the degradation of both dihydrouracil and dihydrothymine to β-ureidopropionic acid and β-ureidoisobutyric acid, respectively (Henderson et al. 1993). The enzymes of the pyrimidine degradation pathway consist of dihydropyrimidine dehydrogenase (DPD), DHP, and β-ureidopropionase (UP). As these enzymes are also involved in the activation and degradation of the widely used antineoplastic drug 5-fluorouracil (5-FU), a deficiency of one of these enzymes has been considered to be clinically important for the risk of severe toxicity after a treatment with 5-FU (Sumi et al. 1998; Van Kuilenburg et al. 2003).

Currently, only 28 patients have been described with DHP deficiency caused by autosomal recessive defects of the *DPYS* gene (OMIM 222748), and 5 of these patients were symptomless individuals who were identified by a screening program (Assmann et al. 1997; Duran et al. 1990, 1991; Hamajima et al. 1998; Henderson et al. 1993; Ohba et al. 1994; Putman et al. 1997; Sumi et al. 1996,

1998; Van Gennip et al. 1997; Van Kuilenburg et al. 2007, 2010). The prevalence of human DHP deficiency in Japan has been estimated to be 1 in 10,000. The clinical phenotype of patients with DHP deficiency was highly variable, ranging from asymptomatic to mental retardation, hypotonia, seizures, growth retardation, dysmorphic features, and gastrointestinal problems. In these patients, a large accumulation of dihydrouracil and dihydrothymine was detected in urine, blood, and cerebrospinal fluid, but in many of these cases, a deficient activity of DHP in liver or kidney tissue was not demonstrated due to difficulties in biopsy and measurement of DHP activity (Assmann et al. 1997; Van Gennip et al. 1997).

Here we report the first case of dihydropyrimidinuria (DHPuria) in a cat possessing a homozygous missense mutation in the feline *DPYS* gene, which is the same as a mutation in human DHPuria.

Subject

A middle-aged stray male cat, looking approximately 4 years old, was sheltered by a caring veterinarian (T.S., one of the authors) since it was suffering from malnutrition. This veterinarian started to feed the cat with a commercial diet as her pet, but found that the animal got sick after eating the food, particularly a high-protein diet, showing lethargy, depression, and vomiting. Laboratory investigations revealed hyperammonemia (135 μmol/L; normal < 44 μmol/L) with normal results of other hematological examinations and serum chemistries. Metabolic substances in urine were analyzed by gas chromatography–mass spectrometry (GC–MS) as reported previously (Song et al. 2008) when the affected cat was approximately 7 years old. The GC–MS demonstrated the presence of large amounts of dihydrouracil and dihydrothymine and moderate amounts of uracil and thymine, suggesting DHP deficiency, whereas these peaks were not observed in unaffected cats. Direct measurement of the activity of DHP, almost exclusively expressed in liver tissue, could not be performed because a liver tissue sample was not available. Currently, the affected cat is at least 10 years of age and being fed a low-protein diet.

Methods

Sequencing Analysis

Genomic DNA extracted from whole blood was used for samples. The feline genomic sequence containing the feline *DPYS* gene was searched for using the Cat BLAST function at the NCBI site based on the exonic sequence of the human (NM_001385.2) and canine (XM_532301.3) *DPYS* gene in the GenBank database. The primers were designed to specifically amplify all coding exons and their adjacent exon–intron boundaries of the feline *DPYS* gene based on the feline genomic sequence database: ACBE01477870 (exon 1), ACBE01477869 (exon 2), ACBE01477868 (exon 3), AANG02147639 (exon 4), ACBE01477865 (exons 5–7), ACBE01477863 (exons 8 and 9), and ACBE01477862 (exon 10), as shown in Table 1. Since information on the downstream sequence of exon 4 was lacking in the GenBank database, RT-PCR was performed for that region using total RNA extracted from leukocytes. The exon numbering of the feline *DPYS* gene was determined based on information from the canine and human *DPYS* genes. The PCR fragments were purified by agarose gel electrophoresis and sequenced in the forward and reverse direction using the BigDye terminator v. 3.1 mix (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

PCR-Primer Introduced Restriction Analysis

To detect the CTT deletion at the 3′-site of intron 5, PCR-primer introduced restriction analysis (PCR-PIRA) was designed with a forward primer generating a site (CTCTTC → CTCTTT) for cleavage by *Ear*I (Table 1), and performed on 26 healthy control cats. The amplification product was digested by *Ear*I and analyzed by electrophoresis in a 3% agarose gel. The 3-bp deletion was judged based on a single 101-bp undigested band whereas a 104-bp amplified band was digested into two fragments, 72- and 32-bp bands, in the wild-type allele.

Population Study of c.1303G>A (p.G435R) Mutation

To investigate the frequency of the c.1303G>A (p.G435R) mutation, a genotyping test using real-time PCR coupled with TaqMan probes was designed according to a previously described method (Chang et al. 2010). The genotype screening was performed on 1,000 mixed-breed cats, blood samples of which were collected in the Kyushu and Shikoku districts in Japan. The primers and probes shown in Table 2 were synthesized by Applied Biosystems. The real-time PCR amplifications were carried out in a final volume of 10 μL consisting of 2× TaqMan GTXpress master mix (Applied Biosystems), 80× TaqMan genotyping assay mix (Applied Biosystems) including specific primers and probes, nuclease-free water, and template DNA.

Table 1 Characteristics of primers and amplified fragments in the direct DNA sequence analysis, RT-PCR, and PCR-primer introduced restriction analysis (PIRA)

Exon	Method	Primer	Sequence 5′ → 3′ (mers)	Location	T_m (°C)	Size (bp)
1	Sequence	Forward	TGGGGTCCCGGAGCCAAG (18)	5′-UTR	62.6	430
		Reverse	GGCTCCGGGGCAGCTG (16)	Intron 1	58.0	
2	Sequence	Forward	AGCAGCGTTGTGCAATTTCG (20)	Intron 1	56.3	354
		Reverse	CACTACCAGTTCCTTGATCTTG (22)	Intron 2	56.7	
3	Sequence	Forward	GTGTGTGTTTGGCTATTGCTC (21)	Intron 2	56.5	338
		Reverse	AGCCACAGAACATTAGTTTCTG (22)	Intron 3	54.8	
4	Sequence	Forward	CTGAAAGCCTGGCCGTGCACT (21)	Intron 3	62.4	288
		Reverse	CGTCTCTCCTCGCATCGGCTA (21)	Exon 4	62.4	
4	RT-PCR	Forward	GAGGGACGAGGAGCTGTATG (20)	Exon 3	60.4	361
		Reverse	GTGCCAGTCTTTATGCCAGTAG (22)	Exon 5	58.6	
5	Sequence	Forward	GATTCCTATCATCCCTCACTAG (22)	Intron 4	56.7	395
		Reverse	GTCAAGTAGAACAGGACACTG (21)	Intron 5	56.5	
6	Sequence	Forward	CTTGAAACAGCTTCTCGTTCTC (22)	Intron 5	56.7	379
		Reverse	AAGCCTTTCTCTGACAGTTTGC (22)	Intron 6	56.7	
7	Sequence	Forward	CTTGCACCTTAATTGATAGGAGA (23)	Intron 6	55.1	289
		Reverse	CATTTCTTTGCAAGTTAGTGCTTC (24)	Intron 7	55.3	
8	Sequence	Forward	CAGATGAGCTGGTGGTGATC (20)	Intron 7	58.4	308
		Reverse	CAGAAACAGGTGACGGTTCA (20)	Intron 8	56.3	
9	Sequence	Forward	GGCACATGAGAGGTAAATTAGC (22)	Intron 8	56.7	397
		Reverse	CTTAGCTAACCTGTCCTTGAG (21)	Intron 9	56.5	
10	Sequence	Forward	GAGTTCTGCATCTCTTTATGAG (22)	Intron 9	54.8	529
		Reverse	AACCAACCTCTCAGAGTCTC (20)	Intron10	56.3	
–	PCR-PIRA	Forward	TGTATTTATCACACACTTTGGTGTGCTCTT (30)	Intron 5	59.2	104
		Reverse	TCCCAAGAGCTTTCTGGCAG (20)	Exon 6	58.4	

Size shows an amplified DNA fragment including primer length

UTR untranslated region, T_m melting temperature; *bp* base pairs

Table 2 Primers and TaqMan probes used for the c.1303G>A (p.G435R) mutation screening

Primer/probe	Sequence (5′ → 3′)	Reporter (5′)	Quencher (3′)	Final concentration (nM)
Forward primer	AACTCATCATCAGGCTGTTAACTTCA	–	–	450
Reverse primer	TGCCTCTGGAAATGGTCACAAG	–	–	450
Wild-type probe	CACCCCGTGGCAAA	VIC	NFQ	100
Mutant-type probe	CACCCTGTGGCAAA	FAM	NFQ	100

VIC 6-carboxyrhodamine, *FAM* 6-carboxyfluorescein, *NFQ* nonfluorescent quencher

Results

Sequence Analysis of the Feline *DPYS* Gene

Exons 1–10 and their exon–intron junctions in the *DPYS* gene were sequenced in the affected cat and the data were registered in the DNA Data Bank of Japan with the accession number AB688984. The sequencing demonstrated the feline *DPYS* gene to be composed of 10 exons including a 1,560-bp putative open reading frame (ORF) from exon 1 to the midstream of exon 9. As shown in Table 3 and Fig. 1, eight alterations were identified: two variations located in noncoding sequences (c.951-7_9delCTT in intron 5 and c.*217T>G in exon 10), five silent mutations in coding sequences (c.51T>C, c.57G>A, c.60G>C, c.492T>C, and c.699C>T in exons 1–4), and one missense mutation (c.1303G>A in exon 8) with an amino acid substitution (p.G435R).

PCR-PIRA for c.951-7_9delCTT

The 3-bp deletion (c.951-7_9delCTT) in intron 5, located 6 bp upstream of the exon–intron splice junction, was investigated in 26 healthy control cats using PCR-PIRA.

Table 3 Results of mutational analysis in the exons and flanking intronic regions of the feline *DPYS* gene

Nucleotide change (exon/intron)	Amino acid change	Genotype	
		Wild-type	Affected
c.51T>C (exon 1)	p.D17D	T/T	C/C
c.57G>A (exon 1)	p.L19L	G/G	A/A
c.60G>C (exon 1)	p.S20S	G/G	C/C
c.492T>C (exon 3)	p.Y164Y	T/T	C/C
c.699C>T (exon 4)	p.A233A	C/C	T/T
c.951-7_9delCTT (intron 5)	–	No del	3-bp del
c.1303G>A (exon 8)	p.G435R	G/G	A/A
c.*217T>G (exon 10)	–	T/T	G/G

The nucleotide and amino acid changes are shown in the affected cat compared to those in the wild-type cat from the GenBank database

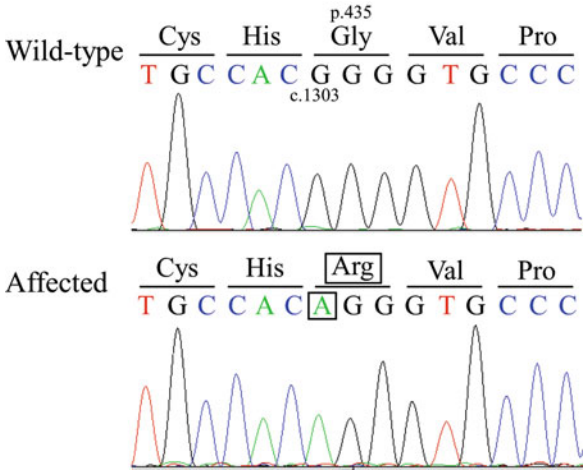

Fig. 1 Partial genomic sequence electropherograms of exon 8 in the feline *DPYS* gene from wild-type and affected cats. The guanine at nucleotide position 1303 is substituted with an adenine in the affected cat. The nucleotide substitution causes the substitution of a glycine at amino acid position 435 with an arginine

As a result, nine cats were homozygous for the deletion and four cats were heterozygotes.

Population Study of c.1303G>A (p.G435R) Mutation

The population study was carried out using the real-time PCR method for c.1303G>A in 1,000 cats. This survey demonstrated that neither heterozygotes nor homozygotes for this mutation were identified and the allele frequency was less than 0.001.

Discussion

The cat in this report had not been given any medication such as 5-FU, but severe clinical signs including lethargy, depression, and vomiting occurred frequently after ingestion of a normal diet, especially a high-protein diet. Due to these unusual recurrent symptoms suggestive of hyperammonemia, a laboratory investigation was performed and an increased concentration of blood ammonia was detected, but results of other hematological examinations and serum chemistries were within normal limits. Since the hyperammonemia without hepatic and renal dysfunction suggested a certain inborn error of metabolism such as urea cycle defects, organic acidemias, and the mitochondrial disorders, GC–MS-based metabolic screening was performed using a urine sample from the cat. The GC–MS analysis demonstrated gross elevations in dihydrouracil and dihydrothymine, that is, DHPuria, as well as moderate elevations in uracil and thymine. These findings strongly suggested that the cat was affected with a DHP deficiency, one of the inborn errors of pyrimidine metabolism, according to the typical GC–MS spectrum in human urine from DHP-deficient patients (Jurecka 2009).

In the cat with DHPuria, activity of DHP has not been measured using liver and/or kidney, the only specimens available for enzymatic confirmation (Assmann et al. 1997; Van Gennip et al. 1997), because of the risk posed by a general anesthesia and difficulty in the measurement of DHP activity. Instead, we analyzed the *DPYS* gene and searched for molecular defects using DNA and RNA from blood of the cat. As a result, the feline *DPYS* gene seemed to be composed of ten exons including 1,560 bp of a putative ORF from exon 1 to the midstream of exon 9, indicating the ORF of the feline gene is the same size as that in the human gene (NM_001385.2). The amino acid sequence homology was 95% similar to the human gene. A significant homozygous missense mutation (c.1303G>A) with an amino acid substitution (p.G435R) was identified in the feline gene of the affected cat (Table 3). The rest of the nucleotide alterations did not seem to be pathogenic because they were silent substitutions without an amino acid substitution in the exonic region and alterations in the untranslated region,

although a possibility of splicing abnormality by these nucleotide alterations is not completely ruled out. The 3-bp deletion (c.951-7_9delCTT) in intron 5 was examined in 26 healthy control cats using PCR-PIRA because this deletion might be related to splicing. However, some of the control cats possessed the deletion homozygously or heterozygously, suggesting it is a polymorphism.

The p.G435R (c.1303G>A) mutation was previously reported in an asymptomatic human patient with DHPuria, although it was found to be compound heterozygous with the p.Q334R mutation (Hamajima et al. 1998). Expression analysis of the human p.G435R mutation in COS-7 cells revealed that it exhibits severely reduced enzymatic activity, demonstrating that p.G435R is a DHP deficiency–causing mutation. In addition, the p.G435R mutant protein was expressed in COS-7 cells at a very low level, although no significant reduction of DHP mRNA expression was observed. However, the residual activity of p.G435R-transfected cells (5.1% of wild-type-transfected cells) was significantly higher than that in the other mutant plasmid-transfected cells, suggesting p.G435R mutation to be one of the less severe types of mutations in humans.

The genotype screening of the feline c.1303G>A mutation revealed no animal to have the mutation in a randomly collected population of 1,000 mixed-breed cats, demonstrating that this mutation is very rare in cats. Based on the result of this screening and the information about the human p.G435R mutation, it is strongly suggested that the cat with DHPuria is affected with DHP deficiency caused by the c.1303G>A (p.G435R) mutation in the feline *DPYS* gene.

In veterinary medicine, usage of 5-FU is contraindicated in cats, but not in dogs, because this drug induces severe neurotoxicity especially in cats (Harvey et al. 1977; Okeda et al. 1988). The scientific reason why 5-FU is severely toxic to cats has yet to be clarified, but it may be due to a lower catabolic rate in the breakdown pathway for pyrimidine bases than other species. Therefore, without any medication such as 5-FU, the affected cat is symptomatic due to the p.G435R mutation, which is less deleterious and does not cause a symptomatic DHP deficiency in humans (Hamajima et al. 1998).

To our knowledge, this is the first case of DHP deficiency in an animal presenting clinical signs in a natural state. Recently, it was demonstrated that human patients with DHP deficiency present mainly with gastrointestinal problems including feeding problems, cyclic vomiting, gastroesophageal reflux, and malabsorption with villous atrophy (Van Kuilenburg et al. 2010), and that seems to agree with the clinical presentation of the affected cat. The cat represents a model of the human disease.

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Synopsis

This is the first report of naturally occurring dihydropyrimidinase deficiency in animals and the cat represents a model of the human disease.

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