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CASE REPORT
Xanthine urolithiasis in a cat: a case report and evaluation of a candidate gene for xanthine dehydrogenase

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Xanthine urolithiasis was found in a 4-year-old spayed female Himalayan cat with a 10-month history of intermittent haematuria and dysuria. Ultrasonographs indicated the existence of several calculi in the bladder that were undetectable by survey radiographic examination. Four bladder stones were removed by cystotomy. The stones were spherical brownish-yellow and their surface was smooth and glossy. Quantitative mineral analysis showed a representative urolith to be composed of more than 95% xanthine. Ultrasonographic examination of the bladder 4.5 months postoperatively indicated the recurrence of urolithiasis. Analysis of purine concentration in urine and blood showed that the cat excreted excessive amounts of xanthine. In order to test the hypothesis that xanthinuria was caused by a homozygote of the inherited mutant allele of a gene responsible for deficiency of enzyme activity in purine degradation pathway, the allele composition of xanthine dehydrogenase (*XDH*) gene (one of the candidate genes for hereditary xanthinuria) was evaluated. The cat with xanthinuria was a heterozygote of the polymorphism. A single nucleotide polymorphism analysis of the cat *XDH* gene strongly indicated that the *XDH* gene of the patient cat was composed of two kinds of alleles and ruled out the hypothesis that the cat inherited the same recessive *XDH* allele suggesting no activity from a single ancestor.

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Xanthine urolithiasis is uncommon in small companion animals (Thumchai et al 1996, Houston et al 2003) and is usually associated with allopurinol administration (Osborne et al 1996, 1999). Therapy with allopurinol causes xanthinuria in animals eating a diet containing purine precursors. However, in a few dogs including Cavalier King Charles spaniels (van Zuilen et al 1997) and Dachshunds (Kucera et al 1997, Flegel et al 1998) that had not been treated with allopurinol, xanthinuria and xanthine urolithiasis have been reported. A family analysis of Cavalier King Charles spaniels with xanthinuria indicated that the disorder was inherited in an autosomal recessive mode (van Zuilen et al 1997). Although several cases of feline xanthine urolithiasis have been documented in cats untreated with allopurinol (Osborne et al 1996, 1999), naturally occurring xanthine urolithiasis has been reported in only one cat (White et al 1997). In the present study, we report a case of xanthine urolithiasis in a cat untreated with allopurinol. In order to scan the gene for causative mutation of a candidate gene, we also evaluate the allele composition of a gene coding xanthine dehydrogenase (XDH) for hereditary xanthinuria.

Case report

A 4.2 kg (9.2 lb) 4-year-old spayed female Himalayan cat was referred to the Veterinary Teaching

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Hospital of Nippon Veterinary and Life Science University with a 10-month history of intermittent haematuria and dysuria. The cat was housed indoors, and was fed a commercially available canned food. The cat was vaccinated periodically. The results of a complete blood count and serum biochemical analysis were within established reference ranges for the laboratory used. Urinalysis using the urine collected by manual expression after abdominal palpation demonstrated haematuria, a pH of 7.0, and a specific gravity of 1.024. No crystals or bacteria were found on sediment examination. Survey of abdominal radiographs showed no apparent reason for haematuria. However, ultrasound examination showed several calculi in the bladder and a thickened bladder wall. A cystotomy was performed because of suspected urolithiasis. Four bladder stones were removed during surgery. The stones were spherical brownish-yellow and their surface was smooth and glossy (Fig 1). Quantitative mineral analysis by infrared spectrophotometry showed that the urolith composition was greater than 95% xanthine (Marpylifetech, Osaka, Japan).

The cat recovered well from the surgical procedure, and was discharged from hospital 3 days after the operation. The cat was fed a canned food with high moisture content in an attempt to reduce urinary xanthine concentration. The cat remained free of clinical signs and 4.5 months later, a follow-up ultrasonographic examination was performed. Surprisingly, several calculi were present in the bladder.

To evaluate abnormalities in purine metabolism, analysis of purine concentration in urine and blood was performed by high performance liquid chromatography (SRL, Tokyo, Japan) (Table 1). When xanthine concentration in urine collected by manual expression was measured in comparison with that of creatinine, the cat was found to excrete an excessive amount of xanthine in urine, and this condition was diagnosed as xanthinuria. The xanthine level was also elevated in blood, and was 18 times higher than the level from healthy control cats. However, the hypoxanthine concentration in the cat's blood sample was significantly lower than that from the control cats. On the other hand, the concentrations of uric acid in both blood and urine were similar between the patient and healthy control cats.

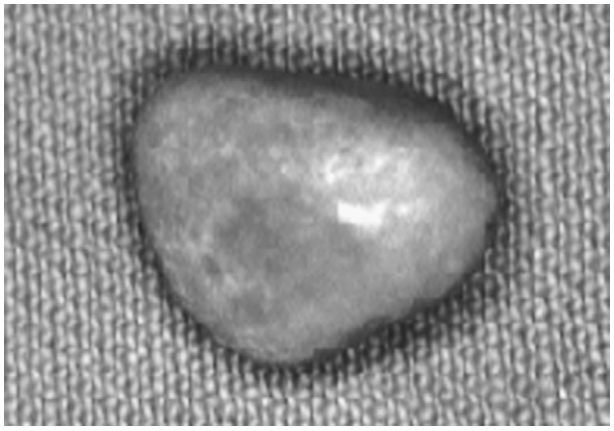

Fig 1. Picture of a urolith containing 95% xanthine.

Table 1. Concentration of purine metabolites in blood and urine from a feline xanthine urolithiasis patient and normal controls

	Patient	Control
Serum		
Hypoxanthine (μM)	4.63	19.02
Xanthine (μM)	12.82	0.70
Uric acid (mg/dl)	0.2	0.17
Urine		
Xanthine/creatinine (μmol/mmol)	63.3	0.072
Uric acid/creatinine (mg/mg)	0.035	0.034

Materials and methods for gene coding

Preparation of genomic DNA from blood samples

Blood samples were collected via venepuncture from the patient with xanthine urolithiasis and 50 control cats. After erythrocytes were lysed in lysis buffer (10 mM Tris (pH 7.5), 0.32 M sucrose, 5 mM $MgCl_2$, 1% TritonX-100), cell pellets were resuspended and incubated overnight at 37°C in an extraction buffer (100 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, 0.5% SDS, 100 μg/ml proteinase K), and were subjected to extraction with phenol and chloroform. The DNA was subsequently precipitated in ethanol. DNA samples were dissolved in TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) and stored at −30°C until use.

Primers for PCR analysis

Primers for PCR amplification of feline *XDH* gene were designed on the sequence of feline

XDH cDNA. After determination of the sequences for counterpart of the human *XDH* gene in the feline *XDH* gene including a DNA fragment from exon 15 to exon 17, feline-specific primers were constructed for amplifying the polymorphic region in feline *XDH* gene. The sequences of the primer pairs were as follows: 5′-AGCTTCTTCTTCAAGGTCGACCT GACAGT-3′ with 5′-GGAATGTCGACACAGTA CACGGCCTC-3′ for feline *XDH* gene from exon 15 to exon 17 and 5′-CAGATTCTAGAG GAAACCACTG-3′ with 5′-CCCGAATTCGCA ACAGTAACAACTGTTC-3′ for the polymorphic region in intron 15 of feline *XDH* gene.

Polymerase chain reaction technique

Polymerase chain reaction (PCR) amplification was performed using 100 ng of genomic DNA samples for the template as described in the manufacturer's protocol. For amplifying the feline *XDH* gene from exon 15 to exon 17 including two introns, PCR was carried out in 20 μl of a reaction mixture consisting of 1× LA PCR buffer (Takara Bio Inc, Ohtsu, Japan), 2.5 mM $MgCl_2$, 0.25 mM each of deoxyribonucleoside triphosphate (dNTPs), 0.2 μM each of primer and 1 unit of *Taq*DNA polymerase (TaKaRa LA Taq™, Takara Bio Inc). The PCR programme involved an initial denaturation step of 2 min at 94°C followed by 30 cycles of two-step reaction of 20 s at 94°C for denaturation and 15 min at 68°C for annealing and extension. For amplification of genomic DNA fragment including polymorphic region in the intron 15 of feline *XDH* gene, PCR was performed in 20 μl of a reaction mixture consisting of 20 mM Tris−HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each of dNTPs, 0.5 μM each of primer and 0.5 units of *Taq*DNA polymerase (Invitrogen Corporation, CA, USA). After an initial denaturation step of 2 min at 94°C, three-step PCR programme was carried out by 35 cycles of 30 s at 94°C for denaturation, 30 s at 58°C for annealing and 40 s at 72°C for extension. The PCR programme was followed by a final extension of 7 min at 72°C and a cooling phase at 4°C. Amplified PCR products were separated by gel electrophoresis in a 2% agarose gel, and were evaluated by ethidium bromide staining and ultraviolet transillumination.

DNA sequencing

After the confirmation by agarose gel electrophoresis, PCR products were purified by a PCR purification kit (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany), and sequenced by dideoxy-mediated chain-termination method with a BigDye terminator kit (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, CA, USA). Sequences were analysed on an ABI Prism 310 apparatus (Applied Biosystems, CA, USA).

Restriction fragment length polymorphism with *Pst*I

After PCR amplifying the DNA fragments of feline *XDH* gene including a polymorphic site in the intron 15, PCR products were treated with restriction enzyme, *Pst*I. Digested fragments were evaluated by agarose gel electrophoresis and ethidium bromide staining.

Results

To evaluate the composition of the *XDH* gene in the cat patient, DNA polymorphisms were investigated in the feline *XDH* gene, and conditions of alleles were analysed. By the PCR method using primers constructed from a feline *XDH* cDNA sequence, a DNA fragment containing a part of feline *XDH* gene was amplified. Sequence analysis of the cloned PCR product showed that the PCR product included a DNA fragment of the counterpart of human *XDH* gene from exon 15 to exon 17 with two introns in feline *XDH* gene (GenBank accession number: DQ097516). Comparison of the DNA sequences of the cloned PCR products from several cats indicated that there was an A−G single nucleotide polymorphism (SNP) in the intron 15 region of feline *XDH* gene (Fig 2A). Three genotypes including homozygous A-type, homozygous G-type and heterozygous A−G-type were observed. The A−G mutation changed the digestion site of the restriction enzyme *Pst*I. The SNP of an A−G mutation was detected by PCR−RFLP using *Pst*I (Fig 2B and C). When the distribution of the A−G polymorphism in the cat *XDH* gene was analysed using 50 unrelated cats, gene frequencies of A-type and G-type alleles were 0.54 and 0.46, respectively (Table 2). This polymorphism was in Hardy−Weinberg equilibrium stating that allele frequencies in a single gene locus remain unchanged after one generation of random mating. The SNP has high variability, which makes it ideal for resolution of allele composition. The cat with xanthinuria was a heterozygote of the A-type and G-type alleles of the polymorphism.

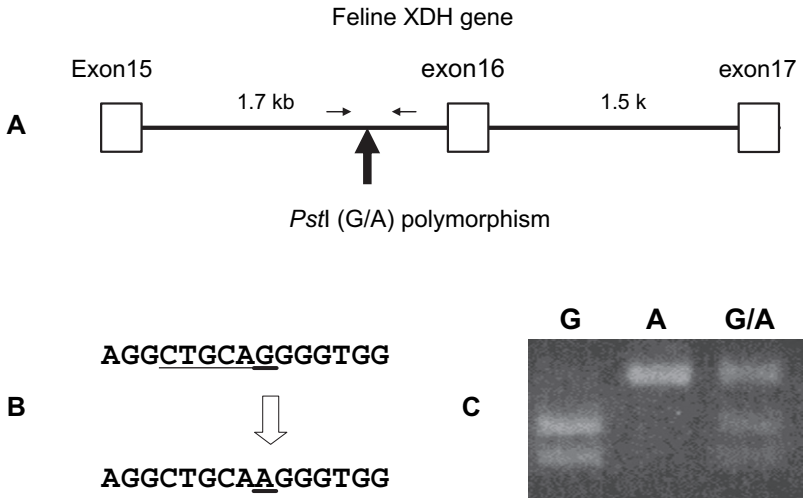

Fig 2. RFLP after *Pst*I digestion in feline *XDH* gene. A: Map of RFLP polymorphic site in feline *XDH* gene. The exons encoding proteins are indicated by open boxes. The number of each exon corresponds to exon in the human *XDH* gene. The lines between open boxes indicate the introns. The number above each line shows the length of the intron. A solid arrow indicates the polymorphic site in the intron of feline *XDH* gene. Two arrows show the primer set used for amplification for PCR-RFLP analysis. B: Localisation of the A−G mutation site. The polymorphic sites are indicated by underlines and an open arrow. A digestion site with *Pst*I is also shown below the G-type sequence in feline *XDH* gene. C: Gel electrophoretic patterns of RFLP after *Pst*I digestion in feline *XDH* gene. Three genotypes of G homozygote, A homozygote and A−G heterozygote were distinguished by digested fragment lengths.

Discussion

There had been no history of allopurinol administration to the cat described in this report. Allopurinol is a competitive inhibitor of XDH, the enzyme catalysing purine metabolism. In this case, four bladder stones estimated by ultrasonographic examination before surgery were removed. It was possible that some calculi were left in bladder or urethra after surgery in spite of repeated flushing and aspiration to remove discrete calculi and small particles of sand for lack of ultrasonographic examination immediately after the surgery. However, recurrence of bladder urolithiasis after surgery and high xanthine concentration in the patient's blood and urine suggested that congenital metabolic abnormalities caused the disease in the cat.

Table 2. Distribution of RFLP with *Pst*I in feline *XDH* gene

Types	No.
G	11
A−G	24
A	15
Total	50

Gene frequencies G: 0.46, A: 0.54 ($\chi^2 = 0.057$, d.f. = 1, $0.80 < P < 0.90$).

Xanthinuria is a rarely recognised disorder characterised by a deficiency in XDH enzyme activity observed in humans and other manmalians. XDH is an enzyme which belongs to the family of molibdo-flavo enzymes that require a molybdenum cofactor and flavin adenine dinucleotide for their catalytic activity along with aldehyde oxidase (AO) and sulphite oxidase (SO) (Simmonds et al 1995, Kisker et al 1997). XDH catalyses two steps of the purine degradation pathway, converting hypoxanthine to xanthine and xanthine to uric acid. XDH deficiency leads to excess urinary excretion of xanthine and hypoxanthine. Hypoxanthine is very soluble, but xanthine is extremely insoluble in urine at any pH (Simmonds et al 1995). Therefore, xanthinuria derived from the deficiency of XDH activity results in the formation of xanthine calculi.

Hereditary xanthinuria in humans is classified into three subtypes according to deficiencies in activities of three enzymes including XDH, AO, and SO (Simmonds et al 1995, Johnson and Wadman 1995). The categories are xanthinuria type I and II, and molybdenum cofactor deficiency. Xanthinuria type I lacks only XDH activity caused by a loss-of-function mutation in the *XDH* gene (Ichida et al 1997). In xanthinuria type II, both XDH

and AO activities are deficient. A mutation in the gene encoding molybdenum cofactor sulphurase (MCSU/HMCS) is responsible for xanthinuria type II (Watanabe et al 2000, Ichida et al 2001). The third type involving molybdenum cofactor deficiency is caused by a loss-of-function mutation of molybdenum cofactor synthetase catalysing the first steps in molybdenum cofactor synthesis (Reiss et al 1998). This type is lacking all three enzyme activities, and is associated with severe neurological disorders in the neonatal period caused by SO deficiency. In the contrary, about 60% of human patients with xanthinuria type I and II (classical xanthinuria) usually have no symptoms and have been discovered incidentally during investigation for another disorder, on routine examination or during investigation in family studies for xanthinuria. Approximately 40% of human patients may develop urinary calculi, acute renal failure or myositis (Simmonds et al 1995). However, in bovine xanthinuria type II especially in Japanese Black cattle, the patient shows lethal growth retardation at approximately 6 months of age (Watanabe et al 2000).

In our study, although there was no information of enzyme activities related to molybdenum cofactor, the cat was diagnosed as classical xanthinuria (xanthinuria I or II), not molybdenum cofactor deficiency, as the cat grew normally and did not have neurological abnormalities. Classical xanthinuria is an autosomal recessive disorder in humans (Simmonds et al 1995). The cat with xanthinuria was thought to have acquired the disorder with xanthinuria because of a homologous mutation in the allele suggesting no XDH activity. In theory, the mutated allele in the *XDH* gene could have derived from one ancestor. However, contrary to our expectations, SNP analysis of the cat *XDH* gene strongly indicated that the *XDH* gene of the patient cat was composed of two kinds of alleles and ruled out the hypothesis that the cat inherited the same recessive *XDH* allele suggesting no activity from a single ancestor. Although it is possible for two kinds of alleles to have no enzyme activity, it is reasonable to think that *XDH* gene mutation is unlikely to be the cause of xanthinuria in this cat. The *XDH* gene is responsible for xanthinuria type I. On the other hand, *MCSU* gene is associated with xanthinuria type II. Xanthinuria type I and II aren't distinguishable clinically without data from an allopurinol loading test or activities of XDH, AO and SO enzymes. If the cat with xanthinuria shows xanthinuria type II, analysis of *MCSU* gene will be useful for determining the cause of the disease.

In the present case, the cat was found to excrete excessive amounts of xanthine in urine, and the blood xanthine concentration was also elevated. According to the purine metabolic pathway in humans, deficient XDH activity not only induces an increase in xanthine levels, but also increases hypoxanthine, and decreases uric acid in both blood and urine (Simmonds et al 1995). The serum hypoxanthine concentration in this cat was significantly lower than that in healthy control cats. Although the hypoxanthine salvage pathway is thought to be enhanced in hereditary xanthinuria patients (Mateos et al 1987), the blood level of hypoxanthine is not below the control level. On the other hand, XDH converts xanthine to uric acid. As a consequence of deficiency of XDH activity, uric acid levels in blood and urine are expected to decrease dramatically. Human patients with classical xanthinuria are usually asymptomatic, and are diagnosed during routine medical examinations with hypouricaemia. However, hypouricaemia was not diagnosed in this cat with xanthinuria. In a previous report in a cat with xanthine urolithiasis (White et al 1997), there was no information on blood and urine examinations for purine metabolism because of accidental death prior to consultation for blood and urine sampling. In a family of Cavalier King Charles spaniels with xanthine calculi, urinary concentrations of xanthine and hypoxanthine were elevated (van Zuilen et al 1997). However, in a Dachshund with naturally occurring xanthine urolithiasis, although xanthine calculi were located in the kidney and bladder, the concentrations of both xanthine and hypoxanthine seemed to be lower than the values in healthy control dogs (Flegel et al 1998). To interpret the oxypurine profile in the present cat, understanding of exact changes in the metabolic pathway involving XDH enzyme deficiency in cats is required.

There is no specific or effective prevention therapy for xanthine urolithiasis. A high fluid intake coupled with a diet low in purine is the only and the most effective prevention treatment for classical xanthinuria in humans (Simmonds et al 1995). In this cat case, only feeding a high moisture food could manage to restrain the growth of bladder stones and the occurrence of clinical signs. To evaluate the effectiveness of a high fluid intake in the prevention therapy in cat xanthinuria, detailed monitoring of xanthine concentration in urine is required in relation to the change of fluid intake.

Feline xanthinuria is recognised to be an extremely uncommon metabolic disorder (Thumchai

et al 1996, Houston et al 2003). It is possible that it is not being detected as inherited xanthinuria because classical xanthinuria usually shows no clinical signs. According to the analysis of mineral composition of feline urolith specimens, 28 of 17,383 were xanthine uroliths (Osborne et al 1999). If all xanthine urolithiases in cats are derived from hereditary XDH deficiency as an autosomal recessive inheritance, although gene frequency of the recessive mutated allele responsible for xanthine urolithiasis is appeared to be exceedingly low in feline population, the distribution of the mutated gene might increase in some inbred cats. Congenital xanthinuria might be one of the inheritary diseases to watch out for in cats.

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