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An *APRT* mutation is strongly associated with and likely causative for 2,8-dihydroxyadenine urolithiasis in dogs[☆]

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Abstract

2,8-Dihydroxyadenine (2,8-DHA) urolithiasis in people is caused by autosomal recessive mutations in the adenine phosphoribosyltransferase gene (*APRT*). 2,8-DHA urolithiasis has recently been reported in two dogs, but, to the authors' knowledge, no studies have yet investigated the genetic basis for susceptibility to the development of 2,8-DHA urolithiasis in this species. Our aim was to sequence *APRT* in dogs affected by 2,8-DHA urolithiasis and compare the results to clinically healthy dogs of similar ancestral lineages. Our hypothesis was that we would identify an autosomal recessive mutation in *APRT* that is associated with the disease. The case population consisted of six dogs with a history of 2,8-DHA urolithiasis: five Native American Indian Dogs (NAIDs) and a mixed breed. The control population consisted of adult NAIDs with no history of urolithiasis. We sequenced *APRT* and identified a missense mutation in a highly conserved codon of *APRT* (c.260G>A; p.Arg87Gln). The c.260A mutation was present in a homozygous state in all six dogs with 2,8-DHA urolithiasis, and it was strongly associated with the disease. This exact missense mutation has been previously reported to cause loss of *APRT* enzyme function in a human cell line, and it is likely a causative mutation in dogs. Therefore, the dog offers a naturally-occurring genetic animal model for 2,8-DHA urolithiasis.

Keywords

2,8-Dihydroxyadenine; Adenine phosphoribosyltransferase; Animal model; Urolithiasis; Genetics

1. Introduction

Adenine phosphoribosyltransferase (*APRT*) is an enzyme involved in the purine salvage pathway, where it catalyzes the conversion of adenine and 5-phosphoribosyl-1-

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Conflict of interest disclosure

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pyrophosphate to adenosine monophosphate [1]. In the absence of APRT, xanthine dehydrogenase (XDH) converts adenine into 2,8-dihydroxyadenine (2,8-DHA), a compound that is highly insoluble in urine. People affected by APRT deficiency (APRTD; OMIM #614723; <http://omim.org/entry/614723>) experience recurrent 2,8-DHA kidney stones and can develop renal damage secondary to renal crystal accumulation [2,3]. APRTD in humans is an inherited disease caused by autosomal recessive loss-of-function mutations in the adenine phosphoribosyltransferase gene (*APRT*) [1–4].

2,8-DHA urolithiasis has been previously reported in two dogs [5,6], but to the authors' knowledge, no studies had been performed to determine the genetic basis for the disease in dogs. We performed a search of the Minnesota Urolith Center's records and identified seven dogs with 2,8-DHA urolith submissions. Of these dogs, five were Native American Indian Dogs (NAIDs), one was described as an Alaskan Malamute mix, and the final dog was called a "wolf." The NAID is a recently founded breed (Fig. 1), derived from the Alaskan Malamute, Siberian Husky, German Shepherd Dog, and Chinook; two dogs from Native American Indian reservations were also reportedly included in the founding population for the breed [7]. The breed trend for this rare stone type supports an underlying genetic basis and motivated this study.

Several urolith types are known to have shared susceptibility genes between dogs and humans. Examples include urate stones and *SLC2A9* mutations [8,9], and cystine stones and *SLC3A1* mutations [10,11]. Logically, the top candidate gene for 2,8-DHA urolithiasis in dogs was *APRT*, the gene implicated in humans with this stone type. Our aim was to sequence *APRT* in dogs affected by 2,8-DHA urolithiasis and compare results to clinically healthy dogs of similar ancestral lineages. Our hypothesis was that dogs with 2,8-DHA urolithiasis share an autosomal recessive mutation in *APRT*.

2. Materials and methods

2.1. Animals

A search of the Minnesota Urolith Center database was performed for canine 2,8-DHA uroliths submitted between January 1st, 1981 and October 1st, 2013. The uroliths were analyzed and confirmed as 2,8-DHA by infrared spectroscopy. Seven canids with this stone type were identified, including five NAIDs, one mixed breed dog (reported to be an Alaskan Malamute mix), and a "wolf." The veterinary clinics that submitted the samples were contacted and asked to request owner participation in the study. Owners for all five of the NAIDs and the mixed breed dog agreed to participate, and DNA samples were obtained from the dogs. Two of the NAIDs were full siblings.

Samples from healthy NAIDs were obtained to serve as a control population with a similar ancestral lineage to the case dogs. Control dogs were required to have no history of urolithiasis or lower urinary tract signs, and they had to be at least 4 years old (selected based on median age of diagnosis of 2,8-DHA urolithiasis in cases). All licensed breeders of NAIDs were contacted to request participation in the study. Two of five NAID breeders agreed to participate. Breeder 1 had not bred any of the case dogs and submitted samples from three control NAIDs of his/her breeding stock. Breeder 2 owned a mating pair that had

produced the two sibling case NAIDs. This breeder submitted samples from his/her entire breeding stock, which included the aforementioned mating pair and six more distantly related control NAIDs. Breeder 2 also contacted owners of full siblings of the two NAIDs from his/her kennel to inform them of the potential problem in the breed and the opportunity to participate in the study. DNA samples from four control NAIDs were submitted from these owners.

DNA samples from dogs of the four breeds (Alaskan Malamute, Siberian Husky, German Shepherd Dog, and Chinook) reported to have served as the foundation of the NAID were also obtained for the purpose of determining the prevalence of any identified mutation(s) in these breeds. These samples came from three sources: 1) dogs owned by faculty, staff, and students at the University of Minnesota Veterinary Medical Center, 2) banked DNA at the Canine Health Information Center (CHIC) database at the University of Missouri, 3) banked DNA at the Canine Genomics Laboratory at the University of Minnesota, and a Chinook breeder. In total, the samples included DNA from 63 Alaskan Malamutes, 58 Siberian Huskies, 61 German Shepherd Dogs, and 3 Chinooks.

Informed consent was obtained from owners of study participants. Samples were obtained directly by owners (cheek swabs), from laboratories (stored DNA), or from veterinarians (blood and tissue) during the course of routine diagnosis and care. Thus, the samples did not fall under a category that requires approval by an Institutional Animal Care and Use Committee.

2.2. APRT sequencing and variant genotyping

Genomic DNA was extracted from whole blood, cheek swabs, or paraffin-embedded tissue using a commercially available kit.¹ Standard Sanger sequencing of *APRT* exons was performed for the six cases with 2,8-DHA urolithiasis and 2 NAID controls. Variants identified in all six case dogs and neither control dog were noted. One of these case variants was a putative functional mutation.

NEBcutter² was used to identify differences in restriction enzyme sites between the reference sequence of *APRT* and the putative functional mutation identified in the case dogs. An enzyme, Hyp188I, was found to recognize and cut the reference sequence but not the variant. The canine reference sequence was used to design primers to amplify an 848 base pair (bp) product encompassing the putative causal variant. Standard PCR amplification was performed with 30 cycles and a 60 °C annealing temperature on a MJ Research PTC-100 thermal cycler.³ The PCR product was incubated with 5 units of the Hyp188I⁴ enzyme at 37 °C for 3 h.

The PCR-RFLP (restriction fragment length polymorphism) assay products were resolved using gel electrophoresis (Fig. 2). Dogs homozygous for the reference nucleotide had 362, 310, and 176 bp products. Dogs homozygous for the variant allele had 538 and 310 bp

¹Puregene blood core kit, Qiagen Sciences, Germantown, MD.

²tools.neb.com/NEBcutter2.

³MJ Research, Inc., Watertown, MA.

⁴New England BioLabs, Ipswich, MA.

products. Dogs heterozygous for the variant had all 4 products (538, 362, 310 and 176 bp). All of the dogs in the study were tested for the putative causal variant with this PCR-RFLP. Samples from two of the dogs that had been directly sequenced, one case (homozygous for the variant) and one control (homozygous for the reference nucleotide), were used as genotype controls for all assays.

2.3. Statistical analysis

Two-tailed Fisher's exact tests were used to compare the frequency of the c.260G>A variant and the proportion of dogs with a homozygous genotype for the variant between case and control groups. A p value of <0.05 was considered significant. The age distribution for the dogs failed the Shapiro–Wilk normality test and is therefore reported as median (range). Analyses were performed with an open source, publically available statistical software.⁵

3. Results

3.1. Phenotypic description of 2,8-DHA urolithiasis cases and NAID controls

All six case dogs were neutered males. As described in Section 2.1, five dogs were NAIDs and one was a mixed breed. Two of the NAID cases were full siblings. The median age of stone diagnosis was 4 (1–10) years. The stones were removed from locations throughout the lower and upper urinary tracts, including the urethra, bladder, ureters, and kidneys. Four of the six dogs had urinary tract obstructions at the time of diagnosis; two had urethral obstructions, one had bilateral ureteral obstructions, and one had both a urethral and a ureteral obstruction. The urolith material was friable and composed of a combination of colors including yellow, green, gray, and black (Fig. 3). The case with bilateral ureteral obstructions was euthanized for renal failure. In addition to the ureteral obstructions, post-mortem histopathology revealed fibrosing interstitial nephritis, glomerular amyloidosis, and brown crystalline material filling the renal pelvis (Fig. 4). An additional dog was diagnosed with chronic kidney disease as a young adult, prior to the discovery of 2,8-DHA urolithiasis, but renal histopathology is not available on this dog.

Allopurinol and a low purine diet were recommended for the five cases that were still living. Four of the cases complied with recommendations. Three had no recurrence of urolithiasis, but the follow-up time was limited (14 months, range 1–28). The fourth dog was noted to have small cystoliths at a recheck ultrasound 8 months after treatment of the original stones. Urine sediment was submitted to the Minnesota Urolith Center and analysis by infrared spectroscopy was consistent with xanthine. The stones were not available to confirm that the composition matched the urine sediment. The fifth case did not follow recommendations and developed recurrence of cystoliths one year after initial diagnosis; the stones were not removed for analysis. After the recurrence, the owner complied with the preventative recommendations, and the dog has not had any further evidence of urolithiasis (20 months follow-up).

⁵R Development Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>.

The 15 NAIDs in the control group were reported to be healthy at the time of study completion and had no history of urinary disease. All control dogs were at least 4 years of age with a median of 5 (4–11) years. Seven of the control dogs were male (five intact and two neutered), and eight were female (six intact and two spayed). As described in Section 2.1, the control group included the sire, dam, and four full siblings of the two sibling NAID cases.

3.2. APRT exonic sequencing results

Exonic sequencing of the *APRT* gene revealed two coding variants present in a homozygous state in all six case dogs. These variants were not found in either NAID control dog. The first variant was in exon 1: c.61G>A resulting in p.Val21Ile. Valine and isoleucine are nonpolar amino acids with similar structures, and comparative analysis with 28 other species demonstrated that isoleucine is a common variant at this codon. Specifically, it is the reference amino acid for the cow, mouse, and rat. Thus, this neutral missense mutation was not evaluated further.

The second variant was in exon 3: c.260G>A resulting in p.Arg87Gln. Arginine is a basic amino acid, whereas glutamine is an amide, and comparative analysis revealed that arginine is conserved at codon 87 in all 28 other species. This non-conservation mutation was considered a putative functional mutation and selected for genotyping in a larger population of dogs.

3.3. c.260G>A genotyping and association results

All six cases were homozygous for the c.260A mutant allele, resulting in an allele frequency in this cohort of 100%. In comparison, only 1 of the 15 NAID controls was homozygous for the mutant allele, 7 were heterozygous, and 7 were homozygous for the reference allele. The overall mutant allele frequency in the control group was 30%. Both the A allele and AA genotype were significantly associated with 2,8-DHA urolithiasis ($p = 7 \times 10^{-5}$ and $p = 1 \times 10^{-4}$, respectively). The 63 Alaskan Malamutes, 58 Siberian Huskies, 61 German Shepherd Dogs, and 3 Chinooks all tested homozygous for the reference c.260G allele.

The single NAID control dog that tested homozygous for the c.260A mutation was a 5 year old intact female dog. The breeder of this dog declined stone screening and urine microscopy to evaluate for 2,8-DHA crystals.

4. Discussion

This study describes an *APRT* mutation in six dogs with 2,8-DHA urolithiasis. The mutation follows an autosomal recessive mode of inheritance with stone risk only observed in homozygous dogs. This is the first report of a genetic basis for 2,8-DHA urolithiasis in dogs and demonstrates that *APRT* is a shared susceptibility gene for urolithiasis in dogs and humans. Based on review of the literature, the dog is the only naturally-occurring animal model for human APRTD.

APRTD is a rare genetic disorder in people with an estimated prevalence of 1:50,000–1:100,000 [1]. The most common manifestation of this disease is recurrent 2,8-DHA

urolithiasis. Acute kidney injury may occur from urinary tract obstruction by calculi, and chronic kidney disease is a common sequela caused by crystalline nephropathy. The age of onset varies, with many affected individuals remaining asymptomatic until adulthood. The kidney stones and damage that occur as a consequence of APRTD can be prevented by treatment with a XDH inhibitor such as allopurinol or Febuxostat (for patients intolerant of allopurinol). Allopurinol diminishes crystalluria, and urine microscopy can be used in treatment monitoring. Dietary purine restriction and high fluid intake are also recommended. These therapies can not only prevent the consequences of APRTD, but they also help dissolve stones and improve kidney function in patients with renal failure [2,3].

The clinical manifestation of disease in the 2,8-DHA case dogs in this study was similar to that observed in humans with APRTD. All six dogs were diagnosed with 2,8-DHA stones as adults (at least 1 year of age). Four of the six dogs presented with obstructive disease; two had urethral obstructions, one experienced both urethral and ureteral obstructions, and one had bilateral ureteral obstructions. Renal histopathology from the dog diagnosed on post-mortem revealed crystalline material in the renal pelvi and chronic renal damage, and a second dog had a history of chronic kidney disease. Allopurinol and a purine restricted diet were recommended for the living dogs. Based on limited follow-up, these therapies may be effective in preventing 2,8-DHA urolithiasis recurrence in dogs with APRTD, as in humans. We do not know the penetrance of the c.260A mutation and lifetime risk for 2,8-DHA urolithiasis in homozygous dogs. However, early initiation of therapy is essential to effective prevention of renal disease in people [2,3]. Therefore, we recommend that even stone-free dogs that test homozygous for the c.260A mutation are started on preventive therapies.

More than 40 mutations in *APRT* have been reported to cause APRTD and 2,8-DHA stone risk in people [1]. The type of mutation varies greatly and includes missense, nonsense, indels, and large deletions. Only individuals with two mutant alleles are affected by the disease. Exonic sequencing of *APRT* in dogs with 2,8-DHA stones identified a missense mutation that results in a shift from a basic amino acid (arginine) to an amide amino acid (glutamine) at position 87 of the protein. Review of 28 other available genomes revealed that arginine is a conserved amino acid at this position across all of the species evaluated, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and multiple plant species. Importantly, a prior study on somatic mutations found that this specific mutation (p.Arg87Gln) occurred in a human cell line and rendered it unable to produce the APRT enzyme [12]. The authors of this study also reported a premature translation termination codon (c.259C>G), an in-frame insertion (c.259_260insGAAAGCCCCA), and a frame shift insertion (c.259_260insCCGA) at codon 87 of *APRT* [12,13]. They concluded that codon 87 is a mutational hot spot in human *APRT*. Our finding of a spontaneous canine mutation at this codon suggests that it may be a mutational hot spot in non-human species as well.

We did not identify the *APRT* mutation in any of the reported foundation breeds for the NAID. There are a few possible explanations for this finding. First, the mutation may have arisen in one of the foundation stock for the NAID and therefore be unique to the breed. An argument against this is that one of the six case dogs was reported to be an Alaskan

Malamute mix. However, this dog was rescued from a shelter, and the lineage may not be accurate. Given the physical similarities between a Malamute and the NAID, it is possible that the mixed breed dog was a true NAID. Alternatively, the mutation may have come from a foundation breed but be present at too low a frequency to detect with the sample sizes used in the study. We tested 58–63 dogs each of the Alaskan Malamute, Siberian Husky, and German Shepherd Dog breeds. When randomly sampling a diploid population, a sample size of 58 is only estimated to capture allele frequencies of at least 5% with a 95% probability [14]. For the Chinook, only 3 dogs were available for study participation. With the small sample size for the Chinook breed, even an allele frequency as high as 50% could be missed.

The greatest limitation of this study was the control group. The control dogs were phenotyped based on the absence of clinical signs of urinary tract disease and an age at or above the median for stone diagnosis in cases. Ideally, the controls would have had a urine sample screened for the presence of 2,8-DHA or an APRT enzyme activity test on hemolysates to rule out subclinical cases of APRTD. While urinary 2,8-DHA levels can be evaluated in human patients, veterinary laboratories do not offer screening for this compound, and we were unable to find a laboratory in the United States to test our canine urine samples. Similarly, the APRT enzyme activity assay is not available or validated in dogs, and we again encountered difficulty identifying a laboratory to test canine samples. Abdominal ultrasonography or contrast studies could have been performed to rule out urolithiasis, but we did not have sufficient funds to cover this testing. Another complicating factor is that half of the control dogs were females compared to none of the case dogs. If the disease penetrance is lower in female dogs, inclusion of this sex in the control group may have resulted in further phenotyping inaccuracies. A single female control dog was homozygous for the variant. This dog is likely an APRTD case, but, as discussed above, phenotyping information was limited. Though we did not have access to assays to measure urinary 2,8-DHA or APRT enzyme activity, we did offer the owner/breeder ultrasonographic screening for subclinical urolithiasis and urine microscopy for 2,8-DHA crystalluria; these tests were declined. Thus, the phenotype of the control dogs may be more appropriately termed “unknown” than “control.” Fortunately, inaccuracies in control phenotyping should increase the risk of a type II error (false negative) but not a type I error (false positive). In other words, the association between the mutation and the disease has likely been underestimated due to these limitations.

5. Conclusion

We identified an autosomal recessive missense mutation in *APRT* that is strongly associated with canine 2,8-DHA urolithiasis. This mutation alters a highly conserved amino acid and has previously been demonstrated to be a loss-of-function mutation in a human cell line. Thus, the mutation is likely causative for 2,8-DHA urolithiasis risk in dogs. Dogs with the *APRT* mutation offer a spontaneous genetic animal model for APRTD and 2,8-DHA urolithiasis.

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Abbreviations

2,8-DHA	2,8-dihydroxyadenine
APRT	adenine phosphoribosyltransferase gene
APRT	adenine phosphoribosyltransferase
APRTD	adenine phosphoribosyltransferase deficiency
NAID	Native American Indian Dog
XDH	xanthine dehydrogenase

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Fig. 1.
Photograph of a Native American Indian Dog. This breed is reported to be derived from the Alaskan Malamute, Siberian Husky, German Shepherd, and Chinook breeds.

Fig. 2.

APRT exon 3 c.260G>A mutation assay using *Hyp188I* restriction digest of an 848 bp PCR product amplified from genomic DNA. Lane 1: Digestion of the reference sequence produces 3 bands (362, 310, and 176 bp). Lane 2: Digestion of sequence from a dog heterozygous for the variant produces 4 bands (538, 362, 310 and 176 bp). Lane 3: Digestion of sequence from a dog homozygous for the variant produces 2 bands (538 and 310 bp). Lane 4: 100 bp ladder.

Fig. 3.
Photograph of canine 2,8-DHA uroliths removed from the lower urinary tract.

Fig. 4. Renal histopathology from a dog with renal failure and bilateral ureteral obstructions with 2,8-DHA urolithiasis. Brown crystalline material is observed in the renal pelvis.