Primary Hyperoxaluria in Cats Is Caused by a Mutation in the Feline GRHPR Gene

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Abstract

Primary hyperoxaluria (PH) is a rare, inherited disease in humans resulting from mutations in the alanine:glyoxylate aminotransferase gene (PH1) or in the glyoxylate reductase (GRHPR) gene (PH2). A disease in cats, mimicking PH2, was described with an autosomal recessive mode of inheritance. Recently, we recognized lesions consistent with PH in kidneys from 3 kittens. Genomic DNA was extracted from 1 blood and 2 formalin-fixed kidney samples from the 3 affected kittens, from blood from the affected cats’ sire, and from blood from 2 healthy unrelated cats. The 9 feline GRHPR exons and intronic donor–acceptor sites were amplified and sequenced. A point mutation G to A was identified at the acceptor site of intron 4. Affected cats were AA, normal cats GG, and the sire was heterozygous A/G. RNA from healthy, carrier, and affected cats was extracted and the GRHPR transcript sequenced revealing an exon 5 deletion in the affected transcript. The 89-bp deletion causes a frameshift and a premature stop codon 44 amino acids downstream, resulting in an anticipated 119 amino acids missing from the C-terminus of the affected cat protein. The unaffected cat expresses the normal transcript, whereas the carrier expressed both.

Key words: feline calcium oxalate, feline genetics, nephrocalcinosis, primary hyperoxaluria

Primary hyperoxaluria (PH) is a rare but potentially fatal inherited disease in humans resulting from mutations in the alanine:glyoxylate aminotransferase (AGT) gene (type I or PH1) or in the glyoxylate reductase (GRHPR) gene (type II or PH2)(Purdue et al. 1990; Cramer et al. 1999; Cregeen et al. 2003). Biochemically, the disease in humans is characterized by decreased hepatic AGT, or GRHPR enzyme activity in type I or type II disease, respectively, increased urinary concentrations of oxalate in both types, as well as increased urinary concentrations of L-glyceric acid in type II. The clinical manifestation of PH is thought to be caused mainly by oxalate accumulation. Increased serum oxalate binds with free calcium ions to form insoluble calcium oxalate deposits. These can subsequently lead to renaloliths, nephrocalcinosis, severe reduction in renal function, and in some severe cases to end-stage renal disease (ESRD) early in life (Leumann and Hoppe 2001). Many patients require dialysis and kidney transplantation, usually in combination with the replacement of the liver. When not treated at an early age and left undetected, PH can lead to serious illness and even death (Leumann and Hoppe 2001).

The genetics of human PH types I and II have been extensively studied with multiple causative mutations identified for each disease inducing dysfunctional AGT and GRHPR protein products (Bobrowski and Langman 2008). The mode of inheritance of the disease is autosomal recessive. The identification of as many causative mutations as possible in humans is important as genetic screening plays a large role in the identification of human patients as well as the identification of silent carriers of the disease (Bobrowski and Langman 2008).

In the late 1980s, a naturally occurring disease in cats, clinically mimicking PH type II, was first described (Blakemore et al. 1988; McKerrell et al. 1989). The disease was seen in a colony of cats with a likely autosomal recessive mode of inheritance. Affected kittens died of acute kidney disease between 5 and 9 months of age. Renal histopathology was consistent with nephrocalcinosis. Increased concentrations of L-glyceric acid and oxalate were observed in
the urine from affected cats, and hepatic GRHPR activity was decreased suggestive of a condition analogous to human hyperoxaluria type II. An additional similar cat was described in a case report in 2005 from Italy (De Lorenzi et al. 2005). Recently, we recognized histopathological lesions consistent with PH in 2 kittens that died from a cat-breeding colony (Figure 1). The goal of this study was to evaluate the feline GRHPR gene as the first candidate gene in the search for the genetic cause of feline PH.

**Materials and Methods**

**Sample Collection**

One blood sample and 2 paraffin-embedded kidney tissue samples were obtained from 3 littermate-affected cats. A blood sample from the affected cats’ sire, who was suspected to be a carrier, was obtained as were blood samples from 2 healthy unrelated adult cats for use in the study.

**Bioinformatics**

Because of the previous report identifying decreased glyoxylate reductase enzyme activity in cats with PH, we hypothesized that a mutation in the GRHPR gene was responsible for the disease in cats. Human mRNA sequence was aligned with the partially assembled feline genome by Blat (http://www.genome.ucsc.edu/) at the University of California, Santa Cruz Genome Browser website. The cat GRHPR exons were hypothesized by investigating the estimated feline sequences for probable splice sites. Nine exons were identified as in the human gene.

**DNA Extraction**

Genomic DNA was extracted from the blood samples as well as from three 5-μm strips, cut from paraffin-embedded tissue blocks, using a commercially available kit (DNeasy Blood and Tissue Kit, QIAGEN Sciences). The amount of renal tissue within each paraffin block varied but approximated 3 × 10 × 10 mm, such that the dimensions of each tissue strip approximated 5 μm × 10 × 10 mm.

**Primer Design, Polymerase Chain Reaction, and Exon Sequencing**

The DNA was amplified exon by exon using specific flanking intronic primers (Table 1). Polymerase chain reaction (PCR) primers flanking each exon were designed using online software (Gene Fisher Interactive PCR Primer Design); http://bibiserv.techfak.uni-bielefeld.de/genefisher/old.html.

PCR reaction volumes were 25 μl using GoTaq Promega Master Mix (Promega Corporation, Madison, WI) and containing 1 μl of DNA from a stock solution (DNA stock solution concentrations ranged from 30 to 70 ng/μl). A 2-stage annealing PCR program on an Eppendorf Mastercycler thermocycler (Eppendorf, Hamburg, Germany) was utilized. PCR settings included the following steps: 1) 5 min denaturation at 96 °C, 2) 20 s at 97 °C, 3) 30 s at 65 °C, 4) 1 min at 72 °C, 5) back to step 2 and repeat for 15 cycles, 6) 20 s at 97 °C, 7) 30 s at 57 °C, 8) 1 min at 72 °C, 9) go to step 6 and repeat for 30 cycles, 10) 5 min at 72 °C, and 11) hold at 4 °C.

Fifteen-microliter samples of the PCR products were loaded into 1% agarose gels made with Tris-Borate-EDTA (TBE) Buffer and stained with ethidium bromide. Samples were submitted for sequencing either after being extracted from the gel (QIAquick Gel Extraction Kit Protocol, QIAGEN Sciences) or directly from the PCR product after purification (QIAquick PCR Purification Kit Protocol, QIAGEN Sciences). Sequencing was performed at the Cornell University Biotechnology Resource Center using the Applied Biosystems Automated 3730 DNA Analyzer (Applied Biosystems, Foster, CA). All exon sequences and flanking intron sequences were examined for polymorphisms that could indicate a disease-carrying mutation.

**RNA Extraction**

Total RNA from healthy and carrier cats’ livers and an affected cat’s kidney (liver was unavailable from affected cats) was extracted from formalin-fixed paraffin-embedded tissue using a previously described method (Chen et al.

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**Figure 1.** Histopathological lesions identified in the renal tissue from a kitten suspected of suffering from PH. Note consistent calcium oxalate crystals in 40× view of hematoxylin and eosin stain (left) and under polarized light (right).
Table 2. Intronic primers used for amplification of GRHPR genomic DNA

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 forward</td>
<td>CCTGTCTCTCTGTGGCA</td>
</tr>
<tr>
<td>Exon 1 reverse</td>
<td>CGCTAGAGACGCGTA</td>
</tr>
<tr>
<td>Exon 2 forward</td>
<td>GAGACGGCTTTTACCTCCTGA</td>
</tr>
<tr>
<td>Exon 2 reverse</td>
<td>CAATGCTGGGCCCCGAG</td>
</tr>
<tr>
<td>Exon 3 forward</td>
<td>CCGCTCTAGTTTCCTGA</td>
</tr>
<tr>
<td>Exon 3 reverse</td>
<td>ACCATGCTGATAGCTGA</td>
</tr>
<tr>
<td>Exon 4 forward</td>
<td>ATTTGGAGATGCCCCAGA</td>
</tr>
<tr>
<td>Exon 4 reverse</td>
<td>TCTGGTTCCCTCCCGTGA</td>
</tr>
<tr>
<td>Exon 5 forward</td>
<td>GGCCGTCTTTACCACTGTA</td>
</tr>
<tr>
<td>Exon 5 reverse</td>
<td>CCCGAGATGCGCCGACA</td>
</tr>
<tr>
<td>Exon 6 forward</td>
<td>TCTATGTTGGGAGCCTGA</td>
</tr>
<tr>
<td>Exon 6 reverse</td>
<td>ATGCTGGCCTTACAGGAA</td>
</tr>
<tr>
<td>Exon 7 forward</td>
<td>CCAATTTGAGGACGACG</td>
</tr>
<tr>
<td>Exon 7 reverse</td>
<td>AAGCTGTGACAGAAGCA</td>
</tr>
<tr>
<td>Exon 8 forward</td>
<td>CCCAGACCTCAGCA</td>
</tr>
<tr>
<td>Exon 8 reverse</td>
<td>AAGCAGACCTCAGCA</td>
</tr>
<tr>
<td>Exon 9 forward</td>
<td>CGGCAACCCCCTGTA</td>
</tr>
<tr>
<td>Exon 9 reverse</td>
<td>CGAGAGATGCCGACA</td>
</tr>
</tbody>
</table>

Results

A total of 5 polymorphisms were identified in the affected cat genomic DNA sequences when compared with the sequences from the normal cats, 4 intronic and 1 exonic. Three of the intronic polymorphisms were found in intron 8. All 3 were single nucleotide polymorphisms that did not appear to segregate with the disease or affect possible splice sites. One exonic polymorphism was identified in exon 8 and represented an A to G change in the affected versus the normal cats located at base number 98153 in scaffold_200192 (March 2006 cat assembly database University of California, Santa Cruz Genome Bioinformatics website: http://www.genome.ucsc.edu/). The subsequent change in codon sequence was analyzed, and the mutation was found to be synonymous, both variants coding for the amino acid serine. The final intronic polymorphism occurred in a hypothesized splice site: A point mutation, G to A, was found at the 3' splice acceptor site of intron 4. Sequencing results uncovered that all affected cats were homozygous for this mutation, whereas all healthy cats were homozygous for the original guanine. The alleged carrier sire displayed the mutation also but was heterozygous for it. (Figure 2).

We hypothesized that the lack of the necessary terminal AG sequence of intron 4 would result in a missplicing event. This would result in the splicing out of exon 5, resulting in a shortened and abnormal mRNA and protein. This hypothesis has been confirmed at the RNA level assessing cDNA PCR products resulting from the reverse transcriptase-PCR of mRNA using exonic primers that span exon 5 of the GRHPR gene (primer pairs were designed within exons 3 and 6 and 4 and 7). The cDNA products of affected cats were shorter than those of normal cats when cDNA was amplified across exon 5 but of normal length when exon 5 was not within the amplicon (primers designed in exons 1 and 4 and 7 and 9). The carrier cat exhibited 1 band similar to the normal cats and 1 similar to the affected cat (Figure 3). Sequencing of the bands purified from the gel confirmed that the difference in the ampiclons is absence of the 89 bp exon 5. The missing nucleotides also result in a reading frameshift causing a premature stop codon to be produced 44 amino acids downstream of the exon 5 deletion site. We would anticipate that this would result in 119 amino acids missing from the C-terminus of the affected cat protein (Figure 4), including the active site of the enzyme (Booth et al. 2006).

Pedigree analysis of the breeding colony database identified the cat that likely introduced the mutant allele to this colony. The litter with the affected kittens was perhaps the first time that a mating had occurred with both the dam and the sire descendents from that same suspected carrier cat.

Discussion

This study analyzing the feline GRHPR gene is the first to investigate the genetic basis of PH type II in cats. In this report, we have identified 5 novel sequence variations in the feline GRHPR gene, 4 of which do not appear to be disease causing and 1 of which does.

The disease-causing mutation, G to A, alters the hypothesized splice acceptor site of intron 4 in diseased...
cats. Mutations in the AG of the splice acceptor site cause missplicing, frequently resulting in exon skipping when the acceptor site of the subsequent intron is then utilized. A similar mutation in the acceptor site of intron 1 in the GRHPR gene in humans followed such a pattern, expelling exon 2 completely from the cDNA (Cregeen et al. 2003). It is likely that this mutation would incur similar consequences and that it plays an important role in causing the disease. We have proved this hypothesis on the RNA level and demonstrated that it is indeed the mRNA of affected cats is shorter and that the sequence confirms the deletion of the 89 bp exon 5 as expected. This deletion in turn induces a frameshift, which results in a premature stop codon and a missing 119 amino acids from the C-terminal of the protein. Based on the structure of the human protein (Booth et al. 2006), this would include the catalytic region of the enzyme and render the affected enzyme nonfunctional. Ideally, we would have liked to confirm the hypothesized effects of this mutation on the structure and function of the GRHPR protein in affected cats as part of this study. Unfortunately, though, we did not have appropriate tissue samples (properly preserved liver or kidney samples) for protein purification or measurement of enzyme activity from the affected cats. The only tissue available from the affected cats was formalin-fixed renal tissue. We believe though that the combination of genomic DNA and RNA evidence is strong enough to make our conclusion as to the cause of the disease in these cats. Purpose breeding of carrier cats will enable us to complete the affected GRHPR protein analysis in the future. The fact that we were able to purify high-quality RNA from the formalin-fixed paraffin-embedded blocks is an adjunct result of this study that opens the door for researchers to attempt additional RNA-based studies on archival tissue that have been thought to not be worthwhile by many in the past.

We considered the search for the causative mutation in cats with PH2 important for a number of reasons despite the fact that naturally occurring disease in cats is thought to be quite rare. First, the disease can be devastating to an inbred cattery where distantly related carriers have been paired producing affected offspring, as occurred in the cattery involved in this study. Today, based on the results of this study, genetic testing can be offered to such a facility that would allow them to discontinue breeding carrier cats and to rid the colony of the disease in a short time. This indeed has been the case in the colony described in this study. It is unclear that feline carriers are completely asymptomatic as is commonly accepted in humans. Due to the extremely high mineral concentrations in cat urine, perhaps even small increases in urinary oxalate concentrations predispose carrier cats with 1 defective GRHPR gene to calcium oxalate stone formation, which is thought to be the most common stone in the kidneys, ureters, and bladders of cats in the United States and in Europe (Cannon et al. 2007; Picavet et al. 2007). Only screening of large numbers of calcium oxalate stone–forming cats will answer the question whether GRHPR gene mutations play a role in this condition other than in the homozygous PH disease form. The third important reason to study this disease in cats is for the potential of feline PH2 to serve as a large animal model in the study of the human disease.

To date, the study of human PH has been challenging due to a variety of causes. These include the rarity of this disease and a large variation in age of onset and clinical presentation. There may also be a vast underdiagnosis of the disease in many patients presenting with kidney stones alone. The fact that, at least until relatively recently and perhaps even today, a definitive diagnosis required a somewhat invasive liver biopsy for enzyme activity assessment (Pais and Assimos 2005) contributed as well.
An additional difficulty in the study of human PH has been the lack of ideal animal models available for researchers to utilize, especially for the study of the pathogenesis and therapeutic aspects of this disease. Available models have traditionally been restricted mainly to 5/6 nephrectomized or ethylene glycol-treated rats (Hatch and Freel 2003). Although these models produced hyperoxaluric rats with chronic kidney disease lasting a few weeks, they differed drastically from the naturally occurring disease seen in human patients. More recently, targeted mutagenesis in the mouse Agxt gene has resulted in a promising new mutant mouse model for PH type I (Salido et al. 2006). Treating these mice with ethylene glycol resulted in severe nephrocalcinosis and kidney disease. This model is promising especially from the aspect of molecular studies and gene therapy, but because of the difference in the course of the disease and the species involved, the opportunities for therapeutic studies are still limited. The establishment of a naturally occurring feline model as discovered in this study for the benefit of human PH research has many advantages over any existing or future rodent models. The ability to assess clinical, biochemical, and histopathological changes in a serial manner, over time, is likely to provide immediate benefit. Studies in pathogenesis and understanding basic principles of PH will benefit from the relative longevity and easy manipulations of the affected cat model. Perhaps, the even larger benefit, though, will be the possibility of future studies surrounding the clinical and treatment aspects of these diseases including stem cell and gene therapy.

References


Figure 4. Alignment of the human GRHPR protein (NP_036335.1), the feline predicted GRHPR protein from a nonaffected cat (cat wt), and the predicted GRHPR protein from an affected cat (cat—). Amino acids different from human are in bold. Note the abnormal and truncated predicted protein of the affected cat.


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