Diagnosis, management and genetic analysis of a cat with primary copper hepatopathy

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Abstract

Case summary A 2-year-old spayed female domestic longhair cat was presented for evaluation of chronic ocular discharge and occasional vomiting. While physical examination findings were consistent with an upper respiratory infection (URI), serum chemistry results revealed increased liver enzyme activities. Histopathologic examination of a liver biopsy identified substantial centrilobular accumulation of copper in hepatocytes – strongly suggestive of primary copper hepatopathy (PCH). Retrospective cytologic examination of a liver aspirate also identified copper aggregates in hepatocytes. After transitioning to a low-copper diet, 1 year of chelation therapy with D-penicillamine achieved normalization of liver enzyme activities and resolution of persistent ocular signs. Subsequently, a long-term regimen of zinc gluconate has been successfully managing the cat's PCH for almost 3 years. Sanger sequencing of the cat's *ATP7B* gene, which encodes a copper-transporting protein, revealed a novel, 'likely pathogenic', single nucleotide variation (c.3670t/a [p.Trp1224Arg]), for which the cat is heterozygous.

Relevance and novel information Recommendations are described for the long-term clinical management of feline PCH – a previously attainable but unreported outcome – with considerations for mitigating the speculated oxidation-exacerbated ocular risks of concurrent URI. This report is the first to include identification of copper aggregates in a liver aspirate from a cat – evidence that liver aspirates from cats could be routinely examined for copper as is standard practice for those from dogs. The cat is also the first reported with PCH and a 'likely pathogenic' heterozygous *ATP7B* genotype, which suggests that normal *ATP7B* alleles could be recessive to or incompletely/co- dominant with deleterious *ATP7B* alleles in cats, as has been reported in other species.

Keywords: Aspirate; *ATP7B*; cornea; cytology; heterozygous; liver; oxidation; rhodamine; Wilson's disease

Case description

A 2-year-old spayed female domestic longhair cat was presented for chronic ocular discharge and occasional vomiting. Since adoption at approximately 8 weeks old, the cat had lived indoors and eaten wet and dry commercially available feline-formulated food. Physical examination identified bilateral watery ocular discharge, nasal planum ulceration, submandibular lymphadenopathy, mildly depressed mentation and pyrexia (103.0°F) – findings consistent with an upper respiratory infection (URI). A complete blood count was unremarkable and feline pancreas-specific lipase was within

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normal limits; however, serum chemistry revealed increased alanine aminotransferase (ALT; 996 U/l, 28–109 U/l) and alkaline phosphatase (ALP; 111 U/l, 11–49 U/l) activities; both were within their respective reference interval before the cat's ovariohysterectomy 18 months prior. Ultrasonographic examination of the abdomen identified diffusely coarse echotexture of the liver and hyperechoic gallbladder walls. The following tests were all negative: fluorescein for corneal ulceration; IgG and IgM titers for *Toxoplasmosis gondii*; PCR for feline infectious peritonitis (sample: liver tissue); and serologic testing for feline leukemia virus (FeLV) antigens, feline immunodeficiency virus (FIV) antibodies and heartworm antigens.

After administration of buprenorphine (0.06 mg/kg, SC) and vitamin K (2 mg/kg, SC, preoperatively; PO, q24h, 30 days postoperatively), anesthesia was induced with alfaxalone (2 mg/kg, titrated to effect over 30 mins) and maintained with inhalant isoflurane throughout an ultrasound-guided Tru-Cut biopsy of the left medial liver lobe. Histopathologic examination of hematoxylin and eosin-stained biopsy sections identified cytoplasmic copper aggregates in hepatocytes with greatest accumulation in centrilobular regions (Figure 1). Subsequent copper-specific rhodanine staining (Figure 2) permitted digital quantification of the copper[1] (dry weight copper = 3051 μg/g, normal <400 μg/g, secondary copper hepatopathy <700 μg/g, primary copper hepatopathy >700 μg/g) and the results were strongly suggestive of primary copper hepatopathy (PCH).[2] A cytologic re-examination of a modified Wright's-stained smear of a liver aspirate identified cytoplasmic blue-green refractile and non-refractile aggregates in hepatocytes – the former consistent with copper and the latter possibly lipofuscin or a copper variant (Figure 3).

To treat the liver disease, metronidazole (20 mg/kg, PO, q12h, 7 days; 10 mg/kg, PO, q12h, 14 days), ursodiol (10 mg/kg, PO, q24h) and Denamarin (18 mg/kg
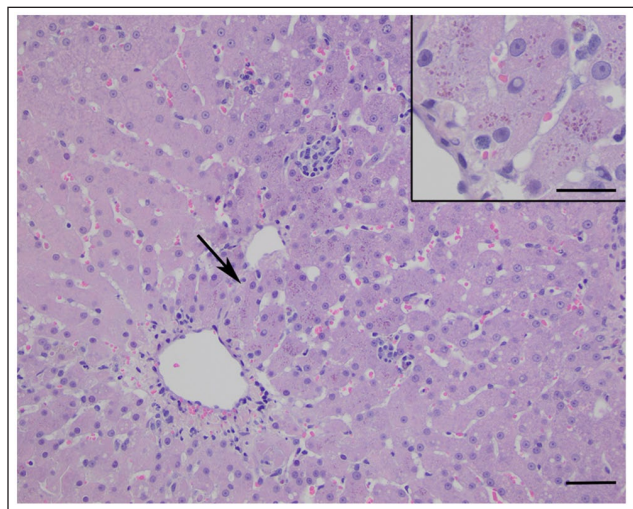

Figure 2 Photomicrograph of a rhodanine-stained section of a liver biopsy from a cat with primary copper hepatopathy. Copper was appreciable as orange-red aggregates around the hepatic veins (HVs) (bar = 1 mm). The inset is a higher magnification image to better demonstrate cytoplasmic copper aggregates in individual hepatocytes (bar = 100 μm)

Figure 1 Photomicrograph of a hematoxylin and eosin-stained section of a liver biopsy from a cat with primary copper hepatopathy. Moderate amounts of cytoplasmic brown-orange pigment (arrow) were evident in hepatocytes, extending from the central vein to the portal region (bar = 50 μm) with most of the pigment found in the centrilobular region. The inset is a higher magnification image of the region closest to the central vein to better demonstrate the cytoplasmic pigment (bar = 25 μm)
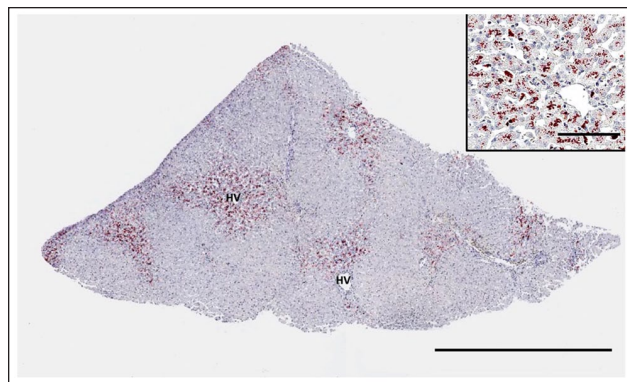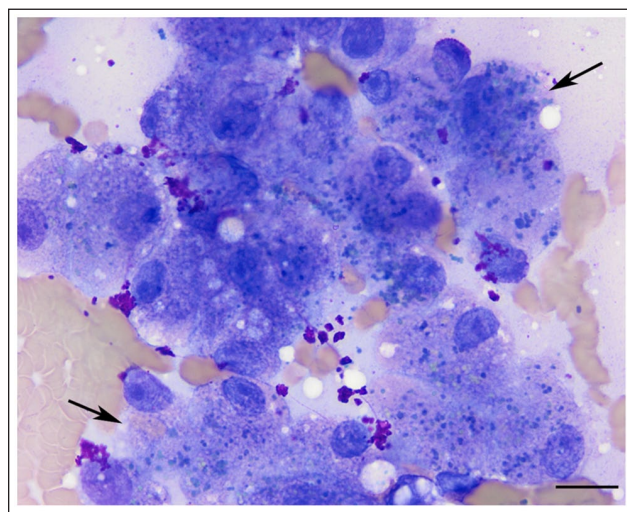

Figure 3 Photomicrograph of a modified Wright's-stained smear of a liver aspirate from a cat with primary copper hepatopathy. The cytoplasm of several hepatocytes contained small amounts of blue-green refractile pigment (arrows), consistent with copper (bar = 10 μm). The more numerous blue-green non-refractile aggregates could be lipofuscin or a copper variant

S-adenosyl-ʟ-methionine and 1.8 mg/kg silybin A+B, PO, q24h) were prescribed. To reduce nausea and hepatic inflammation, maropitant (1 mg/kg, PO, q24h) and prednisolone (1 mg/kg, PO, q12h) were also prescribed. The cat refused a copper-free diet, so commercially available low-copper wet (Salmon and Rice Entrée in Sauce; Purina Pro Plan) and dry (Urinary Care c/d; Hills) foods were fed instead. Despite the administration of zinc gluconate (10 mg/kg, PO, q24h) to reduce enteric copper absorption, liver enzyme activities continued to increase (maximum: ALT >2000 U/l, ALP = 255 U/l), so the zinc gluconate was replaced with D-penicillamine (15 mg/kg, PO, q12h), which chelates copper systemically.

To treat the URI, famciclovir (50 mg/kg, PO, q12h, 14 days) and Clavamox (10 mg/kg amoxicillin trihydrate and 2.5 mg/kg clavulanate potassium, PO, q12h, 5 days) were prescribed. The ocular discharge persisted with intermittent conjunctivitis progressing to corneal opacity and ulceration of the right eye, which was appreciable grossly and highlighted by fluorescein, although corneal cytology was unremarkable. Erythromycin (1 cm of 5 mg/g ointment, OD, q6h, 5 days) was prescribed for its dual antibiotic and anti-inflammatory properties.[3] In addition, intermittent nausea/vomiting and inappetence were treated using fluid therapy (20–40 ml/kg lactated Ringer's solution (LRS) with 37.5 mg/cat B Complex 150 and 0.25 mg/cat B12, SC), mirtazapine (0.4 mg/kg, TD, q48h) and ondansetron (0.2 mg/kg, PO, q12h).

Serial serum and urine chemistries were performed after 1, 3, 11 and 12 months of chelation therapy; minor abnormalities (hyperalbuminemia, hyperbilirubinemia, azotemia and proteinuria) were all characteristic of PCH pathogenesis[2] or treatment.[4] After 12 months of chelation therapy, liver enzyme activities had normalized, and all ocular signs had resolved. Client concerns precluded repeating aspiration or biopsy of the liver, so serum copper was measured and found to be within reference limits (Cu = 0.77 ppm, 0.6–1.4 ppm), suggesting copper homeostasis. However, the lack of a pre-treatment serum copper level for comparison and minimal evidence of correlation between serum and liver copper levels[5] were limitations of this approach. Considering these indicators of copper homeostasis, the cat was transitioned from D-penicillamine back to zinc gluconate as previously prescribed; however, the cat continued to vomit. Decreasing the zinc gluconate dose (6 mg/kg, PO, q24h) substantially reduced vomiting, and the cat's liver enzyme activities have remained within reference limits for almost 3 years, which suggests that this regimen – in combination with the low-copper diet – will effectively manage the PCH in the long term. The cat continues to receive Denamarin, maropitant and ondansetron, as previously prescribed, to support the liver and manage occasional

Table 1 Missense single nucleotide variations (SNVs) identified in the *ATP7B* gene of a cat with primary copper hepatopathy*

SNV (amino acid substitution)	Previously reported phenotype or allele frequency in the 99 Lives population
c.50a>g (p.Asp17Gly)	Healthy
c.1777a>g (p.Thr593Ala)	Healthy
c.1268c/t (p. Thr423Ile) position: chrA1:19,574,548	**C allele frequency = 0.866 T allele frequency = 0.134 267 homozygous C/C 53 heterozygous C/T 19 homozygous T/T 1 unknown**
c.1534a/g (p.Thr512Ala)	Healthy
c.3589g>a (p.Val1197Ile)	Healthy
c.3670t/a (p.Trp1224Arg) position: chrA1:19,609,511	**T allele frequency = 1 A allele frequency = 0 (variation was not called in data set)**

ATP7B genome position: chromosome A1: 19,541,514-19,614,548 forward strand
*Bold text indicates a previously unreported SNV
SNV = single nucleotide variation

vomiting. The cat has remained chronically pyrexic (103.0–105.0°F) in hospital; however, the cat's temperature cannot be taken at home to rule out stress.

Other cases of feline PCH have been linked to variations in copper-transporting ATPase 2,[5,6] a transmembrane protein encoded by the *ATP7B* gene that maintains copper homeostasis by exporting excess cellular copper.[7] Using previously published methods,[5] genomic DNA extracted from the cat's whole blood was Sanger sequenced, and the coding regions of the cat's *ATP7B* gene were aligned with a feline consensus sequence (NCBI: XM_023251165.1) using CLC Sequence Viewer. Alignment identified 23 single nucleotide variations (SNVs), which included six missense variations, two of which had not been previously reported in healthy cats (c.1268c/t and c.3670t/a) (Table 1). Using the 99 Lives Consortium data set (n = 340 cats), c.1268c/t was called with an allele frequency of 0.134, whereas c.3670t/a was not called.

To determine whether these novel SNVs were pathogenic, the consensus amino acid sequence of the feline *ATP7B* gene was aligned with seven other species using previously described methods (Figure 4).[5] Substitutions in functionally important amino acids are more likely to be pathogenic and therefore purged by natural selection (ie, functionally important amino acids are highly conserved across species). While the amino acid affected by the c.1268c/t SNV (p.Thr423Ile) was only conserved

Figure 4 Amino acid sequence alignment of *ATP7B* across eight species in the regions of both previously unknown single nucleotide variations (SNVs) found in a cat with primary copper hepatopathy. The black arrows indicate the locations of amino acid substitutions owing to the novel SNVs. (a) The amino acid affected by the c.1268c/t SNV (p.Thr423Ile) was only conserved across 3/8 species, (b) whereas the amino acid affected by the c.3670t/a SNV (p.Trp1224Arg) was conserved across 8/8 species. NCBI Reference Sequences: dog NP_001020438.1, horse XP_005601246.1, cow XP_002691840.1, pig XP_020920945.1, human NP_000044.2, rat NP_036643.2 and mouse NP_031537.2

Table 2 Protein function analysis of two novel amino acid substitutions found in a cat with primary copper hepatopathy*

SNV	PolyPhen2	SIFT	PROVEAN	PANTHER
c.1268c/t (p.Thr423Ile)	Benign (0.098)	Tolerated (0.44)	Neutral (−0.697)	–
c.3670t/a (p.Trp1224Arg)	Damaging (1.0)	Tolerated (0.07)	Deleterious (−13.291)	Deleterious (−3.7897)

*PolyPhen2 results closer to 1 and SIFT results closer to 0 suggest more pathogenic effects. The c.3670t/a SNV is just slightly above SIFT's 0.05 cutoff for significance. For both PROVEAN and PANTHER, more negative results suggest more pathogenic effects

across three of the eight species (Figure 4a), the amino acid affected by the c.3670t/a SNV (p.Trp1224Arg) was conserved across all eight species (Figure 4b), which suggests that the c.3670t/a SNV and resulting p.Trp1224Arg substitution could be pathogenic.

The effects of both amino acid substitutions on the function of *ATPase 2* were predicted using PolyPhen2, SIFT, PROVEAN and PANTHER, using previously described methods (Table 2).[5] Substantial effects were not predicted for p.Thr423Ile but were predicted for p.Trp1224Arg. The p.Trp1224Arg substitution changes the primary structure of copper-transporting *ATPase 2*'s ATP-binding cytoplasmic domain, which would likely prevent active transport of copper across membranes.

Collectively, these results support conservative characterization of the c.3670t/a SNV as 'likely pathogenic'[8]

(Table 3) and a likely cause of this cat's PCH despite occurring on only one chromosome (ie, the cat is heterozygous for this SNV).

Discussion

Feline PCH is rarely reported[2,5,6,14–16] and has not been as comprehensively studied as in other species. For example, Wilson's disease (WD) is a type of human PCH associated with 1400 known *ATP7B* variations,[10] many more than described from only two sibling[5] and three other cats[6] with PCH. These preceding cats were all homozygous for their respective SNVs, unlike the cat reported here. If this cat is truly heterozygous for this 'likely pathogenic' variation (ie, not compound heterozygous with undetected pathogenic variations, such as in non-coding DNA), then normal feline *ATP7B*

Table 3 Criteria and corresponding evidence for characterizing pathogenicity of the c.3670t/a SNV found in a cat with primary copper hepatopathy

Criterion (from Richards et al[8])	Evidence of pathogenicity for the c.3670t/a (p.Trp1224Arg) SNV
PS1: Same amino acid change as a previously established pathogenic variant regardless of nucleotide change *PM1: Located in a mutational hot spot and/or critical and well-established functional domain (eg, active site of an enzyme) without benign variation* *PM2: Absent from controls (or at extremely low frequency if recessive)* *PP2: Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease* *PP3: Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc)* *PP4: Patient's phenotype or family history is highly specific for a disease with a single genetic etiology*	p.Trp1224Arg has been previously predicted to be pathogenic in humans (see W1153R)[9] but has not been reported in cats c.3670t/a is located in the ATP-binding domain of *ATP7B*, which is a well-established critical functional domain in humans[7] and presumably cats The c.3670t/a variation (A allele) was not called in the 99 Lives data set (n = 340) c.3670t/a is a missense variation in *ATP7B*, where missense variations are a common disease mechanism. Only a few such variations have been reported in cats with many more reported in other species (eg, humans, dogs, mice)[5,6,10–13] PolyPhen2, PROVEAN and PANTHER all predicted pathogenic effects of c.3670t/a. While SIFT predicted that c.3670t/a is 'tolerated,' the numeric result 0.07 is very close to the cutoff for significance (0.05). Additional computational methods predict pathogenicity in humans for p.Trp1224Arg. Also, the Trp residue was evolutionarily conserved across all eight species compared Histopathological sensitivity is high for PCH, which is well-defined in cats. In cats, hepatic copper concentrations >700ug/g with a centrilobular to periportal gradient are highly suggestive of PCH (vs secondary copper hepatopathy),[1] and the *ATP7B* gene is the only reported genetic etiology of feline PCH[5,6]

*While there could be sufficient evidence to characterize the c.3670t/a SNV as 'pathogenic' (1 strong: PS1; 2 moderate: PM1 and PM2; <2 supporting: PP2, PP3 and PP4), some of the evidence requires extrapolation from other species, so the variation was conservatively characterized as 'likely pathogenic' instead. This approach allows for omission of either one moderate and all supporting criteria, both moderate criteria, or the sole strong and one supporting criteria
PCH = primary copper hepatopathy

alleles could either be recessive to or incompletely/co-dominant with deleterious ones. There are also reports of *ATP7B* heterozygous WD patients,[17] yet WD is misleadingly described as a recessive disease. Similarly, *ATP7B* heterozygotes with clinical signs have also been described in laboratory mice,[11] and *ATP7B* heterozygous Dobermans[12] and Labrador Retrievers[13] express an intermediate phenotype. Further research is needed to fully implicate *ATP7B* variations in PCH for non-laboratory species, but the potential for *ATP7B* heterozygotes to be both carriers and clinically ill is evident.

In 50% of WD patients with hepatopathy, copper can also accumulate in the cornea as pathognomonic Kayser–Fleisher rings.[18] While copper was not grossly visible in this cat's corneas, copper-related oxidation was speculated to have contributed to the cat's unusually persistent ocular signs. Oxidative environments are favorable for viruses, especially feline herpesvirus-1 (FHV-1),[19] a common cause of chronic URIs with ocular signs.[20] This cat's coincident return to copper homeostasis and resolution of persistent ocular signs suggests a shared mechanism needing further research. Meanwhile, the potential increased risk of corneal damage from FHV-1 in cats with PCH could be reduced using antioxidant supplements, FHV-1 vaccination and antiviral therapies.

Identifying copper aggregates on cytologic evaluation of liver aspirates from dogs is standard practice, yet evidence of copper aggregates in a liver aspirate from a cat has not been previously published. In modified Wright's-stained smears of liver aspirates, copper aggregates are identifiable as blue-green refractile aggregations within the cytoplasm of hepatocytes.[21] Since copper can be misidentified as lipofuscin, a slightly darker cytoplasmic pigment associated with oxidative damage and aging, additional copper-specific staining, such as rhodanine, is recommended. Identification of copper aggregates in liver aspirates could support recommendations for more invasive and expensive diagnostics (eg, biopsies, genetic testing) in pursuit of a definitive PCH (or other) diagnosis. This case suggests that evaluation for copper could become part of the standard cytologic assessment of liver aspirates from cats.

PCH manifests in young adult cats.[2,5,6,14,16] Before this cat's ovariohysterectomy at 6 months old, all serum chemistry results were within reference limits; therefore, the

PCH was undetectable with routine testing. If this cat had not presented 18 months later for chronic ocular discharge, the occasional vomiting might have been overlooked (likely attributed to longhair cat hairballs) until the cat presented with more significant clinical signs, which have been associated with hepatocellular carcinomas[2] and grave outcomes.[5,14,16] In contrast, this cat's PCH has been well managed for nearly 3 years and is the first reported case with this outcome. Since PCH (and other slowly developing metabolic diseases) may not be detectable on pre-neuter blood panels, routine serum chemistry testing in young adult cats could be used for early detection of these life-threatening diseases.

Conclusions

Serum chemistry testing could be used as an early screening tool for feline PCH, especially in young adult cats. Copper aggregates can be identified on cytologic evaluation of liver aspirates from cats and used to recommend further testing. D-penicillamine chelation therapy can effectively stabilize feline PCH to enable long-term management with zinc gluconate and a low-copper diet. For cats with PCH, ocular signs associated with chronic URIs are speculated to be unusually persistent due to copper-related oxidation of the cornea. Lastly, the heterozygous 'likely pathogenic' *ATP7B* genotype identified in this case of feline PCH suggests that normal feline *ATP7B* alleles could be recessive to or incompletely/co- dominant with deleterious ones.

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