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Case Study

Case Study: Polycystic Livers in a Transgenic Mouse Line

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Three mice (2 male, 1 female; age, 5 to 16 mo) from a mouse line transgenic for keratin 14 (K14)-driven LacZ expression and on an outbred Crl:CD1(ICR) background, were identified as having distended abdomens and livers that were diffusely enlarged by numerous cysts (diameter, 0.1 to 2.0 cm). Histopathology revealed hepatic cysts lined by biliary type epithelium and mild chronic inflammation, and confirmed the absence of parasites. Among 21 related mice, 5 additional affected mice were identified via laparotomy. Breeding of these 5 mice (after 5 mo of age) did not result in any offspring; the K14 mice with polycystic livers failed to reproduce. Affected male mice had degenerative testicular lesions, and their sperm was immotile. Nonpolycystic K14 control male mice bred well, had no testicular lesions, and had appropriate sperm motility. Genetic analysis did not identify an association of this phenotype with the transgene or insertion site.

Abbreviations: K14, keratin 14 promoter; LacZ, bacterial β-galactosidase LacZ reporter; *Lsamp*, mouse limbic system-associated membrane protein.

Polycystic disease is a multiorgan disorder and is the most common genetic life-threatening disease in people, affecting more than 600,000 Americans.[16] Cystic liver disease in people typically is associated with polycystic kidney disease[22,36] but can exist in its absence. Currently, 2 autosomal dominant genes (*PRKCSH* and *SEC63P*) that cause a human polycystic liver disease condition without renal involvement have been identified.[4-6,13]

Numerous rodent models of polycystic kidneys with concurrent polycystic liver exist.[8,34,39,40] However, effective models of polycystic liver without polycystic kidneys would be useful to address clinical and mechanistic issues of polycystic liver not associated with polycystic kidneys.[6,31]

Here we report multiple cases of a spontaneous polycystic liver phenotype without a kidney phenotype in a transgenic mouse line. We also describe the effect of the transgene on disease expression and our attempts to develop this stock as an animal model.

Materials and Methods

Animals. A 16-mo-old female, 6-mo-old male, and a 5-mo-old male keratin 14 promoter–LacZ nonhomologous recombinant (*K14–LacZ*) transgenic mice with grossly distended abdomens due to polycystic liver phenotype were initially characterized. An additional three 5-mo-old male and two 5-mo-old female transgenic mice with polycystic livers were identified via laparotomy.

To generate the transgenic mice, a *K14–LacZ* construct was injected into the pronucleus of Crl:CD1(ICR) embryos for the purpose of labeling tissues that express keratin in periodontal tissue.[26] The strain was maintained for 3 y prior to observation of this phenotype in a small subset of mice. The transgenic mice had LacZ expression in all epithelial cells examined, including biliary epithelium. The Crl:CD1(ICR) mouse stock was chosen as the background stock because it has a high fertility index. The complete nomenclature for this mouse line is CD1–Tg(K14–LacZ)$^{1Rfr(cc)}$.

To determine the significance of transgene insertional mutation, *Lsamp* knockout mice[3] with a targeted deletion of exon 2 of *Lsamp* that were null for *Lsamp* mRNA were obtained (A Pimenta, Vanderbilt University, Nashville, TN). Two male and 2 female homozygous *Lsamp* knockout mice (excluding *Lsamp* as a cystogene) had normal, nonpolycystic kidneys and liver.

Laparotomy. Due to the limited availability of ultrasound equipment on campus, laparotomy was required to assess livers for cysts. We evaluated a total of 21 mice (11 male and 10 female; age, 5 mo), of which 5 mice (3 male and 2 female) had polycystic livers. Mice were anesthetized with 100 mg/kg ketamine (Butler, Dublin, OH) and 5 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA) both given IP. After preparing the skin in an aseptic manner, a 1 to 1.5cm transverse incision was made just caudal to the xyphoid process to visualize the liver. The liver was observed, and the abdomen was closed with muscle and skin layers using 5-0 nylon (Ethicon, Somerville, NJ) and a simple interrupted pattern. The 5 mice with polycystic livers and 2 normal females received buprenorphine (0.2 mg/kg SC) immediately and 15 h after surgery and recovered uneventfully within 2 d after the procedure. All remaining mice with noncystic livers were euthanized by cervical dislocation while anesthetized.

Husbandry. All mice were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*[11] at the University of Illinois at Chicago (Chicago, IL), an AAALAC-accredited institution. All procedures were reviewed and approved by the

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University of Illinois at Chicago Animal Care Committee. Mice were housed in static autoclaved polysulfone microisolation cages (Ancare, Bellmore, NY) with standard irradiated diet (Rodent Diet 7912, Harlan Teklad, Madison, WI), autoclaved municipal water in polysulfone bottles, and autoclaved corncob bedding (7090, Harlan Teklad) on a 14:10-h light:dark cycle.

Microbial surveillance assessments and status. Microbial surveillance was done every 3 mo on female Crl:CD1(ICR) sentinel mice exposed to dirty bedding. Each sentinel mouse cage was exposed to dirty bedding from approximately 50 colony cages. The surveillance program consisted of serologic testing (MFIA Tracking Profile, 3 times annually; MFIA Assessment Plus Profile, once annually; Charles River Labs, Wilmington, MA), fecal flotation, perianal tape tests, and microscopic pelage examination from plucked hair. All the mice were free of mouse parvovirus 1 and 2, minute virus of mice, mouse hepatitis virus, Theiler disease virus, rotavirus, Sendai virus, pneumonia virus of mice, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, *Ectromelia* virus, murine pneumotropic virus, mouse polyoma virus, murine thymic virus, mouse cytomegalovirus, Hantaan virus, *Encephalitozoan cuniculi*, cilia-associated bacillus, ectoparasites, and helminth endoparasites for the past 7 y. This colony had enzootic *Helicobacter* spp. and mouse norovirus.

Additional microbial testing. Special stains (Masson trichrome and Brown and Hopps) and PCR tests (*Helicobacter* spp., mouse norovirus, mouse hepatitis virus, reovirus, *Ectromelia* virus [liver] and minute virus of mice–mouse parvovirus [spleen]; Charles River Labs) were performed to identify bacterial, fungal, and viral agents in the 16-mo-old female and 6-mo-old male mice described following.

Anatomic pathology. Gross and histopathologic analysis was performed on the 16-mo-old female and 6-mo-old male mice with polycystic livers and on five 11-mo-old male and ten 11-mo-old female mice without polycystic livers. Tissues examined histologically included adrenal gland, aorta, bladder, brain, cecum, colon, duodenum, esophagus, eyes, ileum, jejunum, kidney, liver, ovary or testicle, pancreas, pituitary gland, salivary gland, sciatic nerve, secondary sex glands (male mice), skeletal muscle, skin with mammary gland, spinal cord, stomach, thyroid and parathyroid gland, trachea, urinary bladder, and uterus. The testicles and ovaries of 2 mice with polycystic liver and the sole surviving female mouse identified via laparotomy as having a polycystic liver were evaluated histologically.

Clinical pathology. Blood for hematologic and serum chemistry analysis was collected from the 16-mo-old female mouse only. Hematology was performed by using an automated hematology analyzer (Advia 120, Siemens Healthcare Diagnostics, Tarrytown, NY) and Technicon H*1E Multispecies software (version 3, Siemens Healthcare Diagnostics) with mouse-specific algorithms and parameters. Serum chemistry and cystic fluid analysis was performed by using an automated analyzer (ACL 7000, Beckman Coulter, Brea, CA).

***K14–LacZ* gene analysis.** A PCR assay was developed to document the presence of *K14–LacZ* transgene and to determine whether its presence correlated with the polycystic liver phenotype. The sequence of the 5′ and 3′ regions of the transgenic construct was amplified, and the native junctional fragment from the keratin-14 promoter-driven *LacZ* cassette was sequenced. Genomic walking PCR[17] was used to clone the insertion site of the transgene. A *Taq*I generated genomic library was constructed from transgenic mouse DNA, ligated with *Taq*I adaptors, and then amplified by using a primer in the 3′ untranslated region of the K14 transgene (K_14_3PRM_2) and the F_2 adaptor-specific primer. This procedure amplified a 777-bp fragment, which was cloned into pCR2.1-TOPO and sequenced. The fragment contained the 3′ untranslated region of the transgene and 559-bp of flanking mouse genomic DNA.

Another PCR assay identified regions from *Lsamp* to 3′ region of the transgene using primers *Lamp*_F 226555 (TTCACCCAAACCTCAGTGCGG) and KER_R 226557 (CCTCCTGGCAATCAATACAG by using KAPAL polymerase (Takara, Clontech Bio, Mountain View, CA). The PCR conditions were 94 °C for 2 min initial denaturation followed by 30 cycles of 94 °C 20 s, 60 °C for 15 s and 72 °C for 2 min. PCR product was 1.2kb exactly.

Breeding schemes. In an effort to determine whether the polycystic liver phenotype was associated with the K14–LacZ transgene, we established 2 homozygote-to-heterozygote K14–LacZ and 3 heterozygote-to-heterozygote K14–LacZ breeding pairs. The livers from the offspring of the respective breeding pairs were observed visually via laparotomy at 5 mo of age.

In addition, from the five 5-mo-old mice identified via laparotomy to have polycystic livers, one affected male mouse was placed in a cage with the 2 affected female mice; and the remaining 2 male mice were paired with 2 normal female littermates or two 7 wk-old Crl:CD1 (Charles River Labs, Portage, MI) female mice. All were paired 2 wk after recovery from the laparotomy procedure; the incisions of all mice were healed at this time, the mice appeared healthy, and none of the mice had abdominal distention when compared with control mice.

To serve as controls, 5 breeding cages consisting of a 5-mo-old nonpolycystic K14–LacZ male mouse and two 7 wk-old female Crl:CD1 mice (Charles River Labs) were established. The total number of offspring produced from each trio was evaluated for 6 mo.

In an effort to identify additional mice affected with polycystic liver condition, 4 breeder cages of one male and two female heterozygotic K14–β-gal transgenic mice were established. The female mice in each breeder cage were considered to be a group. After both dams in the group each had a litter, the male mouse was rotated to a different group of female mice so that each female mouse had a litter sired by each of the 4 male mice. The rationale behind the breeding scheme was that if the phenotype was polygenic or an autosomal recessive mutation, we would obtain additional affected mice.

Sperm evaluation. In an effort to salvage this line of mice, sperm cryopreservation was attempted in two 11- to 12-mo-old male mice whose polycystic liver phenotype was identified via laparotomy. To serve as controls, 5 nonpolycystic liver mice were evaluated also. Mice for sperm evaluation were euthanized by CO_2 followed by cervical dislocation. The epididymis was minced in M2 mouse embryo culture medium (Millipore, Billerica, MA) and directly examined unstained under a microscope (MZ8, Leica, Wetzlar, Germany) for motility and relative number. The testicles were preserved in 10% neutral buffered formalin for histologic analysis.

Results

Anatomic pathology. *Liver findings.* The predominant finding on gross necropsy of the 3 index cases was a markedly distended liver filled with diffuse myriad 0.1- to 2.0-cm cysts (Figure 1).

Figure 1. *K14–LacZ* transgenic mouse (female; age, 16 mo) showing marked hepatomegaly with myriad 0.1–2.0 cm cysts.

These cysts comprised 50% to 80% of the liver mass. The liver cysts in mice examined via laparotomy were 2 to 3 mm in size at 5 mo and 3 to 15 mm at 10 to 12 mo. Livers were not enlarged on visual examination at 5 mo; livers were larger than normal at 10 to 12 mo, but abdomens were not noticeably distended.

Histologic analysis of the liver in the 16-mo-old female mouse (index case) demonstrated cysts lined primarily with cuboidal cytokeratin-positive epithelial cells with occasional ciliated epithelial cells. Multiple cysts had adjacent focal areas of mild chronic inflammation with mixed inflammatory cells. Connective tissue surrounded some cystic structures. Many portal triads contained multiple biliary profiles that were ectatic, proliferative, and lined by cuboidal to columnar epithelium.

Histologic analysis of the tissues from the 6-mo-old male mouse (index case) showed cysts primarily lined by attenuated epithelial cells with occasional cuboidal or columnar cells (Figure 2). The cystic structures were associated with portal areas. Histologic analysis of the liver from all five 11-mo-old male nonpolycystic K14–LacZ control mice and four of ten 11-mo-old female nonpolycystic K14–LacZ control mice showed mild lymphocytic portal hepatitis.

Testicular findings. In addition to polycystic livers, all of the male mice with polycystic livers that were examined histologically had moderate to severe degeneration of the testicular germinal epithelium. In the 6-mo-old male mouse (index case), the right testicle was of normal size with the epididymis having abundant stores of spermatozoa accompanied by a moderate number of macrophages. The left testicle was approximately 80% of the diameter of the right (normal testicle) and had degeneration of the seminiferous epithelium, much of which was devoid of cells other than Sertoli cells. The epididymis adjacent to this testicle was completely devoid of sperm. Moderate tubular degeneration with aspermatogenesis was noted on histologic examination in both testicles of the male mice with polycystic livers identified via laparotomy that had sperm evaluation. A complete necropsy was not performed on the other index male mouse with polycystic liver. No significant testicular changes were noted in the five 11-mo-old nonpolycystic liver K14–LacZ control mice.

Ovarian findings. Two of the 3 female mice with polycystic livers had either unilateral or bilateral ovarian cysts (diameter, 3 to 5 mm) with moderate parenchymal ovarian atrophy noted histologically. The third female mouse's ovaries were not examined. No significant ovarian changes were noted in the ten 11-mo-old nonpolycystic liver K14–LacZ control mice.

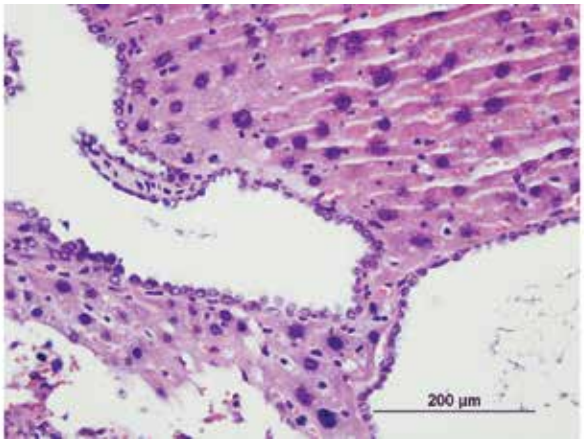

Figure 2. *K14–LacZ* transgenic mouse (male; age, 6 mo) with multiple cysts lined by low, flat epithelial cells and occasionally by cuboidal or columnar cells resembling adjacent biliary epithelium. Hematoxylin and eosin stain.

Additional microbial testing. PCR testing of 2 index cases (the only ones tested) for *Helicobacter* spp., mouse norovirus, mouse hepatitis virus, reovirus, and *Ectromelia* virus (Charles River, Wilmington, MA) did not identify any infectious agents in the liver. Cultures were not performed. All other tests done as part of the microbial surveillance assessment were negative for the past 7 y.

Clinical pathology. Hemoglobin, hematocrit, and albumin values from the 16-mo-old female mouse were decreased (10.8 g/dL, 34%, and 2.36 g/dL, respectively) compared with reference range for the instrument values (11.8 to 14.9 g/dL, 36.6% to 46.6%, and 3.2 to 4.8 g/dL, respectively). Serum liver enzymes ALP, ALT, and AST were elevated (224 U/L, 418 U/L, 624 U/L respectively) compared with the reference values for the instrument (40 to 129 U/L, 10 to 40 U/L, and 10 to 77 U/L, respectively). The cyst fluid had normal electrolyte values when compared with serum reference ranges for the instrument values. Cyst fluid AST was elevated (172 U/L) compared with the reference range for the instrument (10 to 77 U/L); the BUN (23.4 mg/dL), total bilirubin (0.03 mg/dL), and ALP (8 U/L) of the cyst fluid were consistent with reference values for the device.

***K14–LacZ* gene analysis.** The *K14–LacZ* transgene deletes 5 bases (CATAG) at position 41836393 on mouse chromosome 16 (*Mus musculus* strain C57BL/6J chromosome 16, GRCm38.p2 C57BL/6J; http://www.ncbi.nlm.nih.gov/nuccore/NC_000082.6). This is 293,658 bp 3′ from the splice donor of the first exon of the mouse limbic system-associated membrane protein (*Lsamp*) (data not shown). There were at least 2 copies in an array, which was orientated such that the *K14* promoter was driving transcription of the *LacZ* gene in the opposite sense to the *Lsamp* gene. The *Lsamp* gene has a large first intron of 415.8 kb, and the *K14–LacZ* cassette integrated as a tandem array, deleting 5 bp genomic DNA 122.1 kb 5′ of exon 2 of the *Lsamp* gene. Only one transgene insertion site was detected, given that there was a 1-to-1 correlation between results of the *Lsamp* to transgene PCR assay and the PCR assay, which amplifies completely within the boundaries of the transgene.

Breeding schemes. Of the 21 mice examined via laparotomy (13 heterozygotes, 3 homozygotes, and 5 WT), polycystic lesions (maximal diameter, 2 mm) were detected in 5 heterozygote

K14–LacZ mice (3 male and 2 female). These results suggest the polycystic liver phenotype was not associated with the K14–LacZ genotype. The 5 mice with polycystic liver lesions were offspring from a single heterozygote-to-heterozygote breeder cage. The male parent in this breeding pair was necropsied and had a normal liver, but the female mouse died 2 mo previous to the laparoscopic evaluation and was not necropsied.

After 2 mo of breeding, no mice were pregnant despite vaginal plugs having been observed; this pattern is unlike the reproduction typical for normal K14–LacZ mice. Breeder male and female mice were rotated to find a combination that would result in pregnancy, but these breeding schemes demonstrated continued infertility. Within 6 mo after initial pairing, one affected female and one affected male mouse had died. At necropsy, these 2 mice had much larger (maximal diameter, 2 cm) gross polycystic lesions than were noted at laparotomy. From 5 to 11 mo of age, the 5 nonpolycystic K14 male control mice sired 434 pups (38 litters), for a production index of 7.16 pups per month per female mouse.

At necropsy, none of the offspring (age, 4 to 6 wk) that were obtained during follow-up breeding to salvage the mouse line were affected.

Sperm evaluation. The 2 male mice (age, 11 to 12 mo) with the polycystic liver phenotype that were examined had relatively few sperm and no motile sperm even though flagella were noted. All nonpolycystic liver K14 control male mice had numerous sperm, with 50% to 85% of the sperm actively motile.

Discussion

The diagnosis of polycystic livers in our line of transgenic mice was supported by results from gross examination, histopathologic evaluation, and microbial assessment. Cysts examined during laparotomy were small initially (that is, when mice were approximately 5 mo old) but were much larger at the time of euthanasia (that is, 11 to 12 mo of age). This pattern suggests that the condition occurred early in life and was progressive and supports the diagnosis of polycystic liver. Single to few biliary cysts or cystic dilatations of intrahepatic bile ducts are encountered sporadically in aged mice,[10] but multiple cysts throughout the liver are characteristic of polycystic livers and may be relevant to genetically determined polycystic syndromes in humans and animals.[33]

Human hepatic polycystic disease is accompanied by biliary epithelial cell changes consisting of cell proliferation, altered cellular morphology (dysplasia), and fibrosis;[12,32] we noted all of these features in the cases we report herein. In addition, several mice demonstrated peribiliary inflammation, which is common in advanced human cases.[6] We did not obtain samples for culture, but the inflammation was not the primary lesion and special stains and select PCR tests did not demonstrate infectious agents. *Helicobacter* spp. and mouse norovirus were enzootic in this colony, but *Helicobacter* spp. are unlikely to be cleared from the liver so the negative PCR test result is informative.[27] In addition, most *Helicobacter* spp. reside in the posterior intestinal tract. Mouse norovirus has not been reported to affect immunocompetent mice,[27] and the liver was PCR-negative for this agent. However, other undiscovered infectious agents cannot be disregarded entirely. Although cysticercosis is one of the few causes of liver cysts in mice,[1] the morphology of the cysts in our mice was more variable than that expected with cysticercosis, and histopathology confirmed the absence of parasites.

The current case study is the fourth report of polycystic liver phenotype without kidney cysts in a nonhuman mammal, the third report of infertility associated with a polycystic phenotype in a nonhuman mammal, and the first report of a cystic liver phenotype associated with infertility. Polycystic liver without polycystic kidneys has been reported to occur in *orpk* (renamed *Itf88*) transgene rescue mice,[41] in a mutant mouse with a targeted mutation in the polycystic kidney and hepatic disease 1 gene (background strain not indicated),[7] and in 15-mo-old male and female congenic mice (D2.B6[D9Mit90,18]; one of each sex).[24] The infertility in the *wpk* rat model[30] is associated with a very short flagellum that does not extend beyond the cell body; in addition, *wpk* rats have renal cystic dysplasia, encephalocele, polydactyly and biliary dysgenesis. The Oak Ridge Polycystic Kidney (ORPK) mouse is sterile on a FVB/N and C3H background.[15,19,25] ORPK mice typically display a polycystic kidney and liver phenotype, but the kidney phenotype can be differentially rescued by transgenic expression of wildtype *orpk*, thereby offering a potential model to study the liver phenotype.[41]

Polycystic conditions are genetic disorders with heterogeneous etiologies and diverse phenotypic presentations,[21,29] but they most often are caused by genetic defects. The facts that several of our mice with polycystic livers were sired by a single male breeder and that about half of the progeny were normal and half were affected also support this hypothesis.

Lsamp knockout mice were examined to determine whether the polycystic liver phenotype was due to the *LacZ* transgene knocking-out the function of this gene. *Lsamp* is a highly conserved mammalian protein that is characterized as having an adhesion function, a function that is shared with polycystin.[38] The polycystic liver phenotype was not directly related to an insertional mutation caused by the transgene because the *Lsamp* knockout mice did not have a polycystic phenotype. In addition, 5 of the affected mice were progeny of a single breeding pair, suggesting that the phenotype was due to a single spontaneous mutation.

The nature of the genetic defect was not determined, because the mice with this phenotype did not reproduce well. The testicular and ovarian lesions noted in all mice examined histopathologically were most likely the cause of reproductive failure. The testicular lesions were degenerative, in that sperm was not noted on histology; sperm were identified during sperm evaluation likely because this was a larger sample size. Interestingly, the testicular lesion in our initial case was unilateral, which may have been the reason these mice were able to reproduce. The relationship between polycystic livers and infertility in these cases is unclear, but the complete absence of motile sperm could be related to the polycystic liver condition. None of the 5 mice with polycystic livers that we paired at 5 mo of age reproduced, which is markedly different than high production index demonstrated by nonpolycystic K14–LacZ male mice. In addition, the nonpolycystic K14–LacZ mice had highly motile sperm and no testicular lesions. According to the vendor (Charles River Labs), the production index of Crl:CD1 mice is 6 to 10 pups per female per month and is consistent with the production index in the nonpolycystic male K14–LacZ mice in this study. Commercial vendors retire CD1(ICR) mice at 9 to 12 mo of age for production purposes, but mice breed well even after 12 mo.[23]

Abnormal ciliary function and infertility are features of some polycystic syndromes in humans and animals.[2,7,9,20,33,35,37] The role this abnormal function plays in the pathogenesis of polycystic condition has led to the term 'ciliopathies.' In addition, infertility has been reported to occur in human polycystic disease with

a kidney and liver phenotype,[33,35] with polycystins playing a significant role in sperm development and function.[14] Polycystins are highly conserved ubiquitous transmembrane proteins that act as calcium channels into the cell.[28] In addition, they interact with the mechano-sensitive cilia and influence epithelial cell proliferation and cell pressure.[28] The phenotype of concurrent liver cysts and infertility in the absence of kidney cysts that we observed in our mice differs from that in humans—infertility has not been reported in people who have liver cysts.

We noted a polycystic liver phenotype in several mice of the transgenic line. The mice were infertile; contributions of polycystic liver, altered liver function, and exposure to a surgical procedure on infertility were not determined. However, we thought age was of minimal importance, because the nonpolycystic mice bred well and had no testicular lesions. Infertility can limit the acquisition and development of animal disease models and was a limiting factor in the development of a model of polycystic liver disease from this spontaneous event.

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