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A novel cancer syndrome caused by *KCNQ1*-deficiency in the golden Syrian hamster

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Abstract:

BACKGROUND: The golden Syrian hamster is an emerging model organism. To optimize its use, our group has made the first genetically engineered hamsters. One of the first genes that we investigated is *KCNQ1* which encodes for the *KCNQ1* potassium channel and also has been implicated as a tumor suppressor gene.

MATERIALS AND METHODS: We generated *KCNQ1* knockout (KO) hamsters by CRISPR/Cas9-mediated gene targeting and investigated the effects of *KCNQ1*-deficiency on tumorigenesis.

RESULTS: By 70 days of age seven of the eight homozygous *KCNQ1* KOs used in this study began showing signs of distress, and on necropsy six of the seven ill hamsters had visible cancers, including T-cell lymphomas, plasma cell tumors, hemangiosarcomas, and suspect myeloid leukemias.

CONCLUSIONS: None of the hamsters in our colony that were wild-type or heterozygous for *KCNQ1* mutations developed cancers indicating that the cancer phenotype is linked to *KCNQ1*-deficiency. This study is also the first evidence linking *KCNQ1*-deficiency to blood cancers.

Keywords:

Golden Syrian hamster, *KCNQ1*, tumor suppressor

Introduction

The golden Syrian hamster (*Mesocricetus auratus*) is an emerging model organism for human diseases that complements current rodent models. It has been demonstrated to be especially effective in modeling human disorders such as inflammatory myopathies, emerging viral infectious diseases, *Clostridium difficile* infection, pancreatitis, diet-induced obesity, and early atherosclerosis, insulin resistance, dyslipidemia, and virally and chemically induced cancers.^[1,2] However, until recently, a significant challenge for the use of hamsters in modeling human diseases was the inability to generate genetically engineered hamsters. This barrier has recently been surmounted,

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resulting in gene knockouts (KOs) and knockins in the hamster by employing CRISPR/Cas9-mediated gene targeting, *PiggyBac*-mediated transgenesis, and pronuclear injection.^[3,4] This permitted the creation of the first genetic models of cancer in hamsters. One of the first genes that we investigated encodes for the *KCNQ1* potassium channel. *KCNQ1* is widely expressed in human and rodent tissues, including heart, skeletal muscle, smooth muscle, inner ear, renal proximal tubules, gastric parietal cells, exocrine pancreas, intestinal epithelia, lung, hepatocytes, breast, bone marrow, thymic T cells, and white blood cells.^[5-14] *KCNQ1* protein expression data in humans from the human protein atlas^[15] indicates that *KCNQ1* expression is considered high in thyroid, parathyroid and adrenal glands, in stomach and duodenum, and seminal vesicles;

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medium in heart, cerebellum, bone marrow, skeletal and smooth muscle, lung, liver, pancreas, oral mucosa, jejunum and ileum, colorectum, kidney, breast, skin, placenta, and uterus; and low in cerebral cortex, hippocampus, salivary glands, esophagus, testis and ovary (www.proteinatlas.org). KCNQ1 can act as either an S4 domain-containing voltage-gated potassium ion channel or as a constitutively active channel, depending on the identity of its KCNE subfamily heterodimeric partner.^[12,16] KCNQ1 function is best known for its voltage-gated interaction with its KCNE1 heterodimeric partner, such as in cardiac myocyte repolarization.^[12] In contrast, when KCNQ1 partners with KCNE3, as in the intestinal epithelium, KCNE3 locks open the KCNQ1 S4 domain to remove the voltage dependence of KCNQ1 activation, converting it to a constitutively active K⁺ channel.^[12] The *KCNQ1* gene is developmentally imprinted, with its expression controlled by a long noncoding RNA, *KCNQ1ot1*, that lies within exon 10 of the *KCNQ1* gene (and which is itself reciprocally imprinted).^[17] A large number of novel enhancers at the *KCNQ1* locus are reported to regulate the expression of *KCNQ1* in specific tissues^[18] and Wnt/ β -catenin signaling is implicated in the regulation of both *KCNQ1* and *KCNQ1ot1*.^[19-21] *KCNQ1* mutations cause a range of disease in humans such as cardiac arrhythmia (Long QT

syndrome), inner ear defects, and gastric hyperplasia.^[22,23] Germline mutations in humans are associated with disorders called Romano-Ward Syndrome and Jervell and Lange-Nielson Syndrome.^[23] Notably, *KCNQ1* has been shown to act as a tumor suppressor in mouse and human gastrointestinal cancers.^[24-26] In our current study, eight *KCNQ1* homozygous KO hamsters were aged and phenotyped. As early as 70 days of age, seven of these homozygous mutants started showing signs of distress, and on necropsy six of the seven ill hamsters had aggressive visible cancers.

Materials and Methods

Creation of *KCNQ1* knockout hamsters

The *KCNQ1* gene was knocked out in hamsters by CRISPR/Cas9 gene targeting and pronuclear injection following a protocol previously described.^[3,4] Briefly, a sgRNA/Cas9 gene targeting vector designed for hamster *KCNQ1* was constructed using the pX330-U6-Chimeric BB-CBh-hSpCas9 plasmid (Addgene ID: 42230).^[27] The final construct was confirmed by Sanger sequencing. The design of the *KCNQ1* KO allele is depicted in Figure 1a. The 11 bp insertion abolishes a BglI restriction enzyme site, thereby genotyping was carried out by a polymerase chain reaction-restriction fragment length

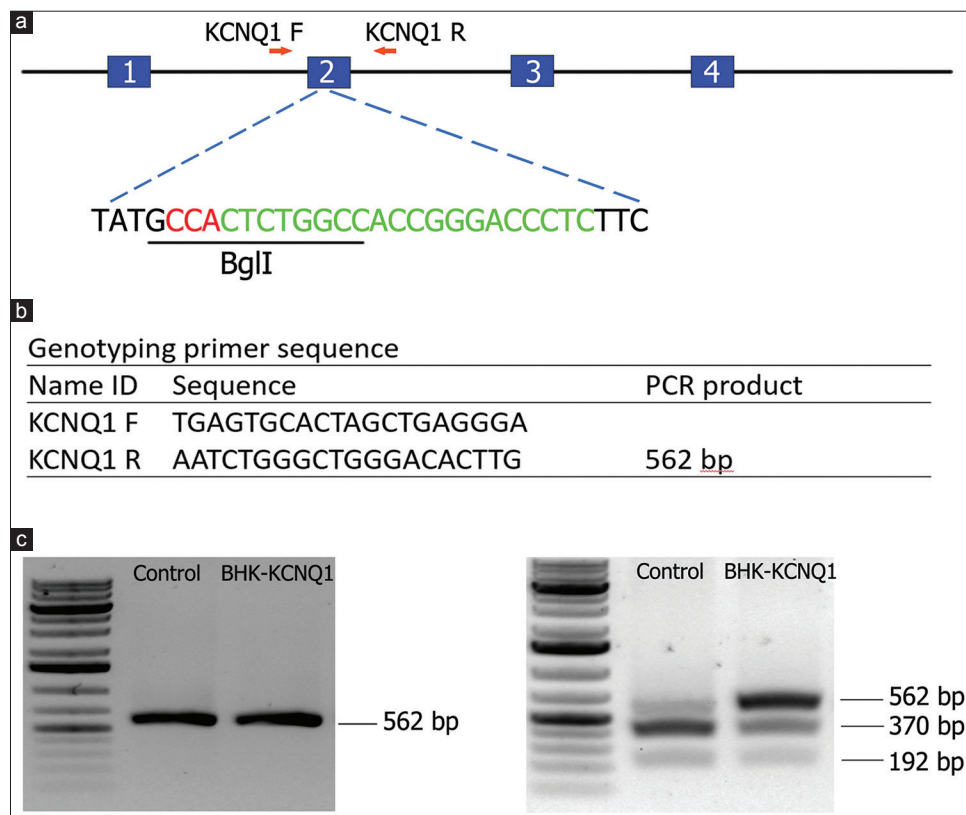


Figure 1: *KCNQ1* gene targeting in golden Syrian hamsters by CRISPR/Cas9. (a) Schematic diagram for the hamster *KCNQ1* genomic locus and the site targeted by the sgRNA. Letters in red are the PAM sequence, letters in green are the sgRNA targeting sequence, and the restriction enzyme recognition site (BglI) used for the polymerase chain reaction-restriction fragment length polymorphism assay is underlined. (b) Primer sequences for genotyping. (c) Gel images from the baby hamster kidney cell transfection assay results indicating the success of the design and genotyping and efficiency before pronuclear injection. Left, uncut with BglI; right, cut with BglI

polymorphism (PCR-RFLP) assay. PCR primers are listed in Figure 1b. To determine the success of the design, genotyping and targeting efficiency, gene targeting was conducted in baby hamster kidney (BHK) cells. BHK fibroblasts (BHK; American Type Culture Collection, Manassas, VA, USA) were cultured in Minimum Essential Medium supplemented with 10% fetal bovine serum, nonessential amino acids, and Penicillin-Streptomycin (Life Technologies, Carlsbad, CA, USA). A volume of 5 mg of circular sgRNA/Cas9 vectors were transfected into 106 BHK cells using Amaxa 4D-Nucleofector (Program No. CA-137; Lonza, Allendale, NJ, USA). Two days posttransfection, cells were harvested for genomic DNA isolation using Puregene Core Kit A (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Target DNA was PCR amplified from the genomic DNA isolated from BHK cells using Phusion High-fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA), [Figure 1c] (left). After digestion with BglI, the PCR products were resolved on a 1% agarose gel and stained with SYBR green dye (Life Technologies, Carlsbad, CA, USA) [Figure 1c] (right). To determine gene targeting efficiency, the relative intensities of uncut band and cut bands were analyzed using the Image J software (1.47p, NIH). To produce *KCNQ1* KO hamsters, we microinjected the sgRNA/Cas9 gene targeting vector into the pronuclei of hamster zygotes, followed by transferring the injected hamster embryos to recipient females and allowed the resultant pregnant females to naturally deliver and raise the pups. Genotyping analysis of the F0 pups was done by PCR-RFLP assays,

and the genotyping results for F0 pups are shown in Figure 2a. To reveal the nature of the indels introduced, genomic PCR products used in the PCR-RFLP assays for each of the pups were subcloned into TA cloning vectors and sequenced by Sanger sequencing. To establish a *KCNQ1* KO breeding colony, we bred F0F7 (female; carrying both +1nt and +11nt indels) with a WT male. Genotyping analysis with the PCR-RFLP assays showed that both the +1nt and +11nt indels transmitted to the germline [Figure 2b]. Genotyping results by the PCR-RFLP assay on F2 pups produced sister-brother breeding between two heterozygous F1 pups (#6 and #8) [Figure 2c], both carrying the +11nt allele, and produced three homozygous KO (#1, 2, and 3), 7 heterozygous KO (#4, 5, 8, 10, 11, 12, and 13) and four WT (#6, 7, 9, and 14) F2 pups [Figure 2d]. To identify potential off-targets, we did blast searches with the sgRNA coding sequence as the query against the hamster genome (https://uswest.ensembl.org/Mesocricetus_auratus/Info/Index). Top potential off-targets for sgRNAs are listed in Table 1. The corresponding DNA oligos for each of the targeting sites were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA). We performed PCR-RFLP assays for each of these genomic loci but did not identify any off-targeting events. *KCNQ1* homozygous KO hamsters demonstrate similar neurological defects (unpublished data from our group) as KO mice,^[25] including deafness, inner ear defects, head bobbing, and smaller stature. For this reason, breeding required crosses between heterozygous animals. Hamster histopathology on formalin-fixed tissues was conducted by two trained pathologists at

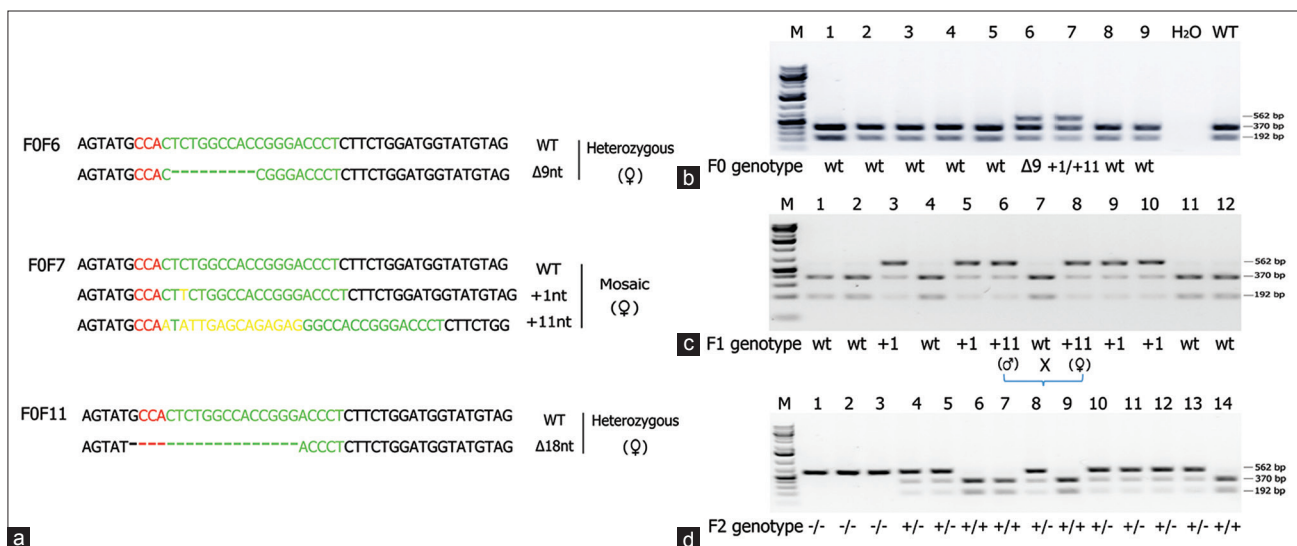


Figure 2: Establishment of a breeding colony of *KCNQ1* knockout hamsters. (a) Indels introduced into *KCNQ1* in 3 F0 founder hamsters. wild-type; Δnt and + nt: nucleotide deletions and insertions, respectively. The +11nt allele in FOF7 is the result from the replacement of a CT dinucleotides by 13 random nucleotides. (b) Genotyping result of the PCR-RFLP assay on a litter of F0 founder hamsters. FOF6 carries a $\Delta 9nt$ indel and FOF7 carries both +1nt and +11nt indels. (c) Genotyping results with the PCR-RFLP assay on F1 pups produced from breeding an FOF7 (female) with a WT male. Indels in each of the targeted pups were revealed by Sanger sequencing. (d) Genotyping results by the polymerase chain reaction-restriction fragment length polymorphism assay on F2 pups produced from sister-brother breeding between two heterozygous F1 pups (#6 and #8) both carry a +11nt allele from the FOF7 founder as indicated in C)

Table 1: Top 5 potential off-target sites for sgRNA/Cas9-KCNQ1

Site ID	Sequence*	NCBI sequence ID	Location of site
KQOff1	c AGGGT Ct GGTGGCCAGAG TGG	NW_004801604.1	Range 1: 71859496 to 71859518
KQOff2	GA A G c T I CCGGTGGCCAGAG CAG	NW_004801605.1	Range 1: 32864166 to 32864188
KQOff3	a AG t T C CC a GTGGCCAGAG CAG	NW_004801647.1	Range 1: 8649479 to 8649501
KQOff4	cct GGT Ct GGTGGCCAGAG TGG	NW_004801613.1	Range 1: 24582636 to 24582658
KQOff5	c AGG t Ct CGGTGGCCAGAG TGG	NW_004801644.1	Range 1: 7417525 to 7417547

*PAM is indicated in bold and italic letters. Mismatches between the target and the potential off-target sequences are in red and lower-case. NCBI: National Center for Biotechnology Information

our institutions pathology core facility. All animal work was approved by the Institutional Animal Care and Use Committees at our universities.

Results

Cancers in *KCNQ1* knockout hamsters

Seven of the eight homozygous mutants developed severe physical distress beginning at ~70 days of age, including six hamsters with overt cancers at necropsy. The cancers were synchronous, often large and infiltrative to multiple organs – liver, lung, pancreas and omentum, intestine, kidney, spleen, sternum, lymph nodes, and stomach in addition to systemic myeloproliferative disease involving bone marrow, lung, liver, and spleen. The major cancers observed were T-cell lymphomas, plasma cell tumors, and hemangiosarcomas (HAS). None of the 48 hamsters in our colony that were wild-type or heterozygous KO for *KCNQ1* developed cancers indicating that the cancer phenotype is linked to *KCNQ1*-deficiency. Heterozygous animals were also generally phenotypically normal. As shown in Table 2, a detailed description of all hamsters including gross and microscopic pathology findings and diagnoses.

T-cell lymphomas

T-cell lymphomas were the most common cancers observed in hamsters. Three hamsters were diagnosed with abdominal masses and T-cell lymphoma involving liver, spleen, kidney, small intestine, colon, pancreas and omentum, based on histopathology and anti-CD3 immunohistochemistry [Figure 3].

Plasma cell tumors

Plasma cell neoplasms, similar to multiple myeloma in humans, were diagnosed in two hamsters by histopathology and methyl green-pyronin staining, and found in liver, pancreas, spleen, omentum, lymph nodes, and mesentery. In one hamster, a plasma cell tumor mass measuring 4 cm × 3 cm × 3 cm engulfed the liver, pancreas, spleen, and associated lymph nodes and local mesentery. A prominent lymphoid population (suspect lymphoma) was found co-infiltrating the plasma cell tumor in these animals. Plasma cells were also found co-infiltrating tissues with T-cell lymphoma [Figure 4].

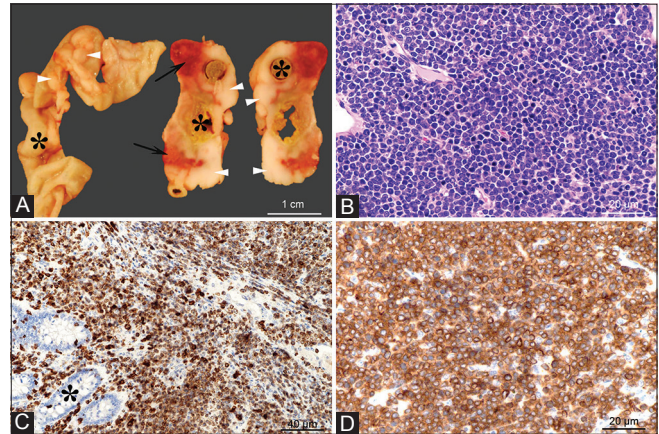


Figure 3: Gross and microscopic images of an intestinal T-cell lymphoma. Grossly (Panel A), extending from the intestinal wall through the serosa and mesentery, there are multifocal to coalescing white-beige masses (arrowheads), with focal areas of necrosis and hemorrhage (arrows). The tumors circumferentially surround the intestines (indicated by asterisks) which are occasionally moderately dilated and segmentally ulcerated (transmural). Histologically, the tumor consists of densely packed sheets of round to oval cells with scant basophilic cytoplasm (Panel A) that infiltrate the intestinal mucosa (Panel C, asterisk) and subjacent layers through to intestinal serosa. The cells show a uniform, intense immunolabeling of plasma membrane for CD3 (Panels C and D) (anti-CD3 antibody, DAKO/Agilent, Santa Clara, CA, USA; Cat# A0452). H and E, ×40 objective Panel B; CD3 immunolabeling with ×20 objective Panel C, and with ×40 objective Panel D

Hemangiosarcoma

HSA, while a common cancer in canines,^[28] is rare in humans and rodents. Based on pathology, we observed multifocal HSA in the lungs and HSA and hemangiomas in the liver of two hamsters [Figure 5].

Myeloid disease and other suspect cancers and findings

Myeloproliferative disease was observed in four animals (with possible myeloid leukemia in one animal) involving bone marrow, lung, liver, and spleen; this involved multiple lineages, notably, the granulocytic series (heterophil, sometimes eosinophil), and in addition plasma cells, megakaryocytes and red cells to varying degrees. In one hamster, a prominent myeloid/polymorphonuclear population was observed in the bone marrow that was also infiltrating the adjacent fibrous connective tissue of the sternum and striated muscle. One hamster was diagnosed with granulosa cell tumor involving the ovaries.

Table 2: List of all hamsters

Hamster #	KCNQ1 genotype	Sex	Age (d) at death	Gross pathology findings/comments	Key diagnoses
21759	-/-	Female	88	A large, globoid mass, 4 × 3 × 3 cm, partially replaces the left lobe of the liver, is heterogeneous, rubbery, with whitish and pale-beige, slightly friable areas. The visceral lymph nodes and mesentery around the pancreas are diffusely thickened	Plasma cell tumor involving liver with prominent CD3 + cell population/possible lymphoma Hemangiomas, multiple, liver Myeloproliferation (multilineage) involving liver, pancreas, omentum, and lymph node (coeliac lymph center)
21760	-/-	Female	139	Large 3 × 2 × 2 cm mass involving most of the left part of the abdomen, encircling intestine and tubular organs. Liver has multiple small dark foci. Lungs have several small 1-3 mm dark nodules involving several lobes	Abdominal T-cell lymphoma encasing intestines and uterus, and infiltrating/involving liver, spleen, and intestine, kidney (confirmed by IHC using anti-CD3 staining) Myeloproliferation (multilineage) or possible myeloid leukemia involving bone marrow, lung, liver, and spleen Hemangiosarcoma, multicentric involving lungs and liver Ovary: Granulosa cell tumor
21761	-/-	Male	130	A 1.5 × 1.5 × 1.5 cm, homogenous white-beige, infiltrative mass involves the liver. The spleen is diffusely enlarged (marked splenomegaly), with prominent white pulp. Bilaterally the kidneys are encased in thin, white-beige, infiltrative mass	Abdominal T-cell lymphoma involving the liver, spleen, (confirmed by IHC using anti CD3 staining), omentum, and lymph node (coeliac lymph center) Plasma cell tumor surrounding and infiltrating kidney Myeloproliferation (multilineage) involving liver, pancreas, kidney and omentum
21762	-/-	Male	160	Found dead in cage, large white mass attached to some internal organs, tissue necrosis, pathology not feasible	Suspect hematopoietic neoplasm
21701	-/-	Male	227	Persistent diarrhea marked weight loss	Not examined
21703	-/-	Female	238	Large irregular mass ~ 3 × 3 × 2 cm, white-tan, firm, focally necrotic involving the right abdominal wall, mesentery and omentum, and encasing intestines, uterine serosa	Abdominal T-cell lymphoma encasing intestines and involving the uterine serosa, mesentery, and abdominal wall (confirmed by IHC using anti-CD3 staining) Myeloproliferation (multilineage) involving bone marrow, liver, spleen and lung
21758	-/-	Female	70	Found dead in cage, bloated, large white necrotic mass involving internal organs, pathology not feasible	Suspect lymphoma or plasma cell tumor
21702	-/-	Male		Alive at 360 days	None
21740	+/-	Male		Alive at 339 days - breeder	None
21742	+/-	Female		Alive at 335 days - breeder	None
21774	+/-	Female		Alive at 196 days - breeder	None
21778	+/-	Male		Alive at 196 days - breeder	None
29 other hamsters	+/-	Male + Female		29 other hamsters used in breeding, ages ranged from 40-370 days, euthanized	None
15 other hamsters	+/+	Male + Female		15 other hamsters used in breeding, ages ranged from 40-175 days, euthanized	None

IHC: Immunohistochemistry; +: wildtype; -: knockout; ~: approximately; #: hamster identification number

Discussion

To the best of our knowledge, this study represents the creation of the first genetically engineered hamster cancer model. Our work is also the first evidence linking *KCNQ1*-deficiency to blood cancers. How might *KCNQ1*-deficiency cause blood cancers in hamsters? In humans and mice, *KCNQ1* has been reported to be expressed in several types of hematopoietic cells, including thymic T cells,^[14] bone marrow (www.proteinatlas.org), and murine white blood cells,^[13] although *KCNQ1* expression patterns in

hamsters have not been fully characterized. There is also a report that disruption of *KCNQ1* in mice results in an accumulation of mature T cells.^[14] One mechanistic possibility is that *KCNQ1* may synergize with hamster polyomavirus (HaPV). HaPV is thought to be endemic in hamster colonies worldwide (including ours), and an outbreak of lymphomas was reported in a colony in Spain of the genetic audiogenic seizure-prone hamster: Salamanca strain of hamsters susceptible to audiogenic seizures.^[29] Notably, studies of this colony have suggested that the seizure phenotype may be linked to a deficiency for the nonvoltage-gated *KCC2* potassium

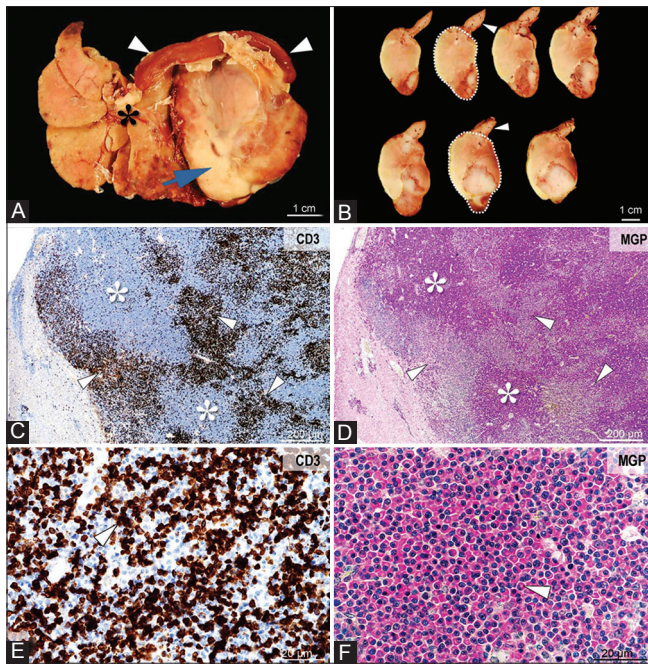


Figure 4: Gross and microscopic images of lymphoma and a plasma cell tumor involving the liver. Grossly, the tumor (Panel A, arrow) infiltrates and displaces the liver (Panel A, asterisk) and spleen (Panel A, arrowhead). On cross-section, the tumor (Panel B, demarked area) is heterogeneous, white-beige, friable, with large areas of necrosis and hemorrhage (Panel B, arrowheads). Histologically, the tumor consists of large areas of neoplastic plasma cells (Panel C and D, asterisks), associated with coalescing foci of CD3 immunopositive lymphocytes (Panel C-E, arrowheads). The densely packed plasmacytoid cells are round to oval, with variably sized, and eccentrically placed nuclei with coarse chromatin, a prominent Golgi area, and cytoplasm that stain intensely for methyl green-pyronin (Panel F, arrowhead). CD3 immunolabeling, objective $\times 4$ (Panel C) and $\times 40$ (Panel E). Methyl green-pyronin stain for plasma cells, objective $\times 4$ (Panel D) and $\times 40$ (Panel F)

channel in the brain.^[30] Thus, there is the possibility that a potassium channel deficiency may cooperate with HaPV in cancer development. More specifically, deficiency for the voltage-gated KCNQ1 potassium channel could cause membrane depolarization in bone marrow-derived stem cells, contributing to their transformation. A role for KCNQ1 in regulation of stem cell transformation has been proposed for KCNQ1-deficiency in gastrointestinal cancer, arising from bidirectional Wnt/ β -catenin signaling.^[21] Consistent with this, in GI cancer KCNQ1 expression was linked to the regulation of intestinal stem cell mRNA in mice,^[25] and KCNQ1 protein expression has been localized to the intestinal stem cell compartment of intestinal crypts (unpublished work by our group). Further support for a role in stem cells is a report of KCNQ1 expression in a murine bone marrow-derived pluripotent small embryonic/epiblast-derived stem cell Very small embryonic-like stem cells.^[31] Future studies will need to analyze whether Wnt/ β -catenin signaling is dysregulated in KCNQ1-deficient bone marrow-derived hamster progenitor cells, especially with the emerging evidence of a major role for Wnt signaling in hematological malignancies.^[32] Finally, the observation of HAS in addition to neoplasia involving

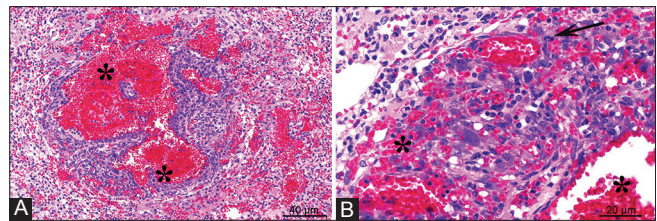


Figure 5: Histopathological images of a locally infiltrative multicentric hemangiosarcoma involving lung. Tumor composed of pleomorphic, oval to spindle-shaped cells that form irregularly shaped, blood-field vascular spaces (asterisks Panels A and B), with occasional atypical mitotic figures (Panel B, arrow). H and E, $\times 20$ objective Panel A, $\times 40$ objective Panel B

several hematopoietic cell lineages in these hamsters is interesting, and appears to be consistent with the finding of this tumor in dogs and its attribution to transformation of a common progenitor hematopoietic cell.^[28]

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Nil.

Conflicts of interest

There are no conflicts of interest.

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