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A FAS-ligand variant associated with autoimmune lymphoproliferative syndrome in cats

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Abstract British shorthair (BSH) kittens in multiple litters died as a result of a severe non-neoplastic lymphoproliferative disease that showed many similarities with human autoimmune lymphoproliferative syndrome (ALPS). Human ALPS is caused by inherited defects in FAS-mediated lymphocyte apoptosis and the possibility of similar defects was investigated in BSH cats. The whole genomes of two affected kittens were sequenced and compared to 82 existing cat genomes. Both BSH kittens had homozygous insertions of an adenine within exon 3 of the *FAS-ligand* gene. The resultant frameshift and premature stop codon were predicted to result in a severely truncated protein that is unlikely to be able to activate FAS. Three additional affected BSH kittens were homozygous for the variant, while 11 of 16 unaffected, but closely related, BSH cats were heterozygous for the variant. All BSH cats in the study were from a population with significant inbreeding. The variant was not identified in a further survey of 510 non-BSH cats. Identification of a genetic defect in the FAS-mediated apoptosis pathway confirms that the lymphoproliferative disease in BSH cats fulfills the diagnostic criteria for ALPS in humans. These results will enable the development of a genetic test to detect BSH carrier animals.

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a rare non-neoplastic lymphoproliferative disease typically seen in infants or young children with diverse racial backgrounds (Sneller et al. 2003). As the majority of the patients remain undiagnosed or misdiagnosed, the incidence and prevalence of the disease is currently unknown (Rao and Oliveira 2011). However, the phenotype has been detected and shown to be segregating in over 300 families worldwide (Rao and Oliveira 2011). The lymphoproliferation results in marked lymphadenopathy and splenomegaly with autoimmune cytopenias often present in affected individuals (Worth et al. 2006). The majority of patients with ALPS have germline defects in the *FAS* gene, although defects in the *FAS ligand* (*FASL*) or *caspase 10* can also cause ALPS (Del-Rey et al. 2006; Nabhani et al. 2014; Rieux-Laucat et al. 2003; Sobh et al. 2016). Any of these defects reduces the body's ability to trigger FAS-mediated lymphocyte apoptosis resulting in an accumulation of T-lymphocytes that expand lymph nodes and the spleen (Fisher et al. 1995). Patients with ALPS are predisposed to developing malignancies with 10–20 % of affected individuals subsequently developing lymphoma (Worth et al. 2006). The majority of human ALPS cases have an autosomal dominant pattern of inheritance, but the genotype often shows incomplete penetrance and the resulting phenotype is highly variable (Teachey et al. 2010).

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Recently, three of five kittens from a litter of British shorthair (BSH) cats bred in New Zealand (NZ) developed marked widespread lymphadenopathy at around 6 weeks of age (Aberdein et al. 2015). The kittens were all from a relatively isolated population that had a history of inbreeding. Consistent with ALPS, the lymphadenopathy was due to a non-neoplastic proliferation of CD3+/CD4−/CD8− 'double negative (DNT)' T-lymphocytes (Aberdein et al. 2015). While an ALPS-like disease was suspected, demonstrating defective apoptosis or identification of a variant in a gene coding for a protein in the apoptosis pathway is necessary to fulfill the diagnostic criteria for ALPS (Oliveira et al. 2010). Three more affected litters were subsequently identified in NZ BSH cats, and pedigree analysis was consistent with an inherited disorder (Aberdein et al. 2015). The observation that multiple BSH kittens in Australia had also developed a similar non-neoplastic lymphoproliferative disease (R. Malik, unpublished data) provided additional evidence of a genetic variant present within this breed of cat.

The complete genomic sequences of cats have already been used to identify causal variants for clinically defined genetic diseases, such as identification of the causal *COLQ* variant in Devon Rex cats with congenital myasthenic syndrome (Gandolfi et al. 2015) and the identification of an *AIPL1* variant causing progressive retinal atrophy in Persian cats (Lyons et al. 2016). The aim of the present study was to use complete genomic sequencing to identify the variants present in two affected BSH cats. The identification of a genetic variant in a gene involved in the FAS pathway in the affected BSH cats would strongly support a genetically mediated defect in the FAS pathway and fulfill the diagnostic criteria for an ALPS-like disease in these cats. Identification of the causative variant will permit development of a genetic test to identify carriers, avoiding any mating that could lead to expression of the disease in the BSH population.

Materials and methods

Sample collection and whole genome sequencing

The WGS of two BSH cats that developed early onset marked lymphadenopathy were determined as part of the 99 Lives Cat Genome Sequencing Initiative (www.felinegenetics.missouri.edu/99Lives). Briefly, DNA was extracted from frozen samples of liver and kidney that had previously been preserved in RNAlater solution (Thermo Fisher Scientific, Waltham MA) using a Qiagen MagAttract HMW DNA Kit (Qiagen, Hilden, Germany), and concentrated using a DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA) according to the manufacturers' instructions. Whole genome sequencing was performed as previously described by producing two PCR-free libraries and generating ~30× genome coverage from 100 paired-end reads on an Illumina HiSeq 2500 (Illumina, San Diego, CA) at the University of Missouri DNA Core (Gandolfi et al. 2015). Generated sequences were processed and the variants were analyzed using active filters provided by Maverix Biomics, Inc. (Santa Cruz, CA) as previously described (Gandolfi et al. 2015). Briefly, reads were demultiplexed and trimmed using TRIMMOMATIC (Bolger et al. 2014) and aligned using BWA-MEM (Li and Durbin 2009) to the *Felis catus 6.2* genome (htpp://www.ncbi.nlm.nih.gov/assembly/320798). Variants were identified using PLATYPUS (Rimmer et al. 2014) and variant effects and impacts were predicted using SNPEFF (Cingolani et al. 2012) (http://snpeff.sourceforge.net/SnpEff_manual.html#eff). The variants were filtered by selecting only variants within the whole genome that were homozygous in both affected cats and not present in any of the other 72 domestic and nine wild felid sequences that existed at the time in the 99 Lives sequence database. The predicted effect of the identified variants on protein structures was modeled using Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index).

To identify variants in archived material from earlier NZ cases, DNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples of kidney from three additional affected BSH kittens and a FFPE kidney sample of an unaffected control domestic shorthair cat. DNA was also extracted from blood that had been archived from the sire and the dam of the first litter of kittens with this disease. All DNA was isolated using a Roche high-pure FFPE Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Blood samples were collected from 14 BSH cats from an Australian cattery that had previously produced litters that included kittens that developed ALPS-like disease to evaluate the presence of the suspected variant in a larger number of cats and breeding lines. These cats were selected by the breeder as potentially carrying the variant. DNA was extracted from the blood using a Qiagen DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions.

FASL genotyping

To detect the presence of the variant in *FASL* in the archived samples from NZ cats, specific primers were designed to amplify a 173 bp region of DNA that contained the variant using Geneious 8.1 software (Biomatters Ltd, Auckland, NZ; Table 1). Amplification conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. The final extension was

Table 1 PCR primers used for genotyping *FASL* variant in cats

Assay	5′ Primer (5′-3′)	3′ Primer (5′-3′)
Sequencing of New Zealand samples	tgacctgttcagagtcccaac	aaggctttcccaaaccccat
Sequencing of Australian samples	cagtgccacccaagttctaagc	accagaaagtaacaactccctcagag
Genotyping (Massarray)	tacaggtcaactcaatccac	gtaccttccagatacagacc
Genotyping Massarray extension	ccgtctcagctccctttttt	

at 72 °C for 5 min. Amplified DNA was purified from a 1.5 % agarose gel containing ethidium bromide using a Qiaquick PCR purification kit (Qiagen) and submitted for automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and using a ABI3730 Genetic Analyzer (Applied Biosystems Inc, California, USA). The presence of the variant within DNA extracted from blood samples taken from Australian cats was investigated using different specific primers that amplified a 659 bp fragment (Table 1). PCR amplification, purification, and sequencing were performed as described previously (Gandolfi et al. 2015).

Population screening

Archival DNA samples from 510 non-BSH cats of 29 different non-BSH breeds and random bred cats were screened using mass spectrometry (MassARRAY, Agena Bioscience, San Diego, CA). An assay was designed to type the identified variant (Table 1), and genotyping was performed on the samples using the Agena Bioscience iPLEX Gold Genotyping reagent set. Products were typed with the MassARRAY System with Nanodispenser RS1000 (Agena).

Results

The two cats selected for WGS were siblings from a single litter. Both kittens developed massive lymphadenopathy due to a marked polyclonal expansion of T lymphocytes at 8 weeks of age (Fig. 1a, b). Approximately 20× genome coverage was produced for each of the two cats. The polymorphisms in the BSH cats were identified by comparison to 82 unaffected cats in the 99 Lives WGS variant database. Genome wide, including all polymorphisms homozygous and unique in the two affected BSH cats, 3233 variants were identified after active filtering, distributed as follows: 1 high impact variant, 14 moderate impact variants, 9 low impact variants, and 3209 modifiers variants. The 15 non-synonymous variants unique to the two BSH WGS and therefore concordant with the disease (Table 2) were evaluated. While fourteen missense variants were in different genes, as described in Table 2, only one

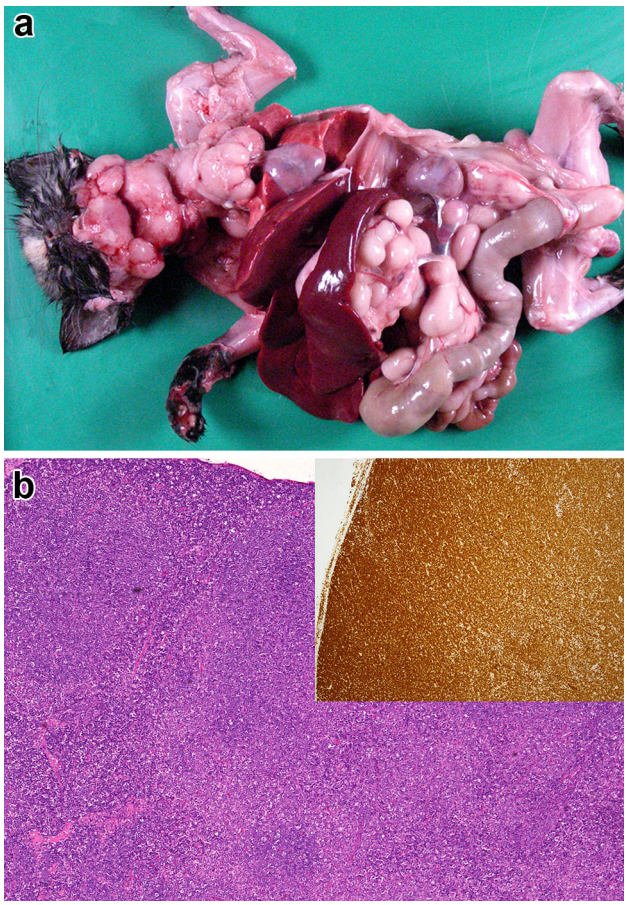

Fig. 1 **a** An 8-week-old British shorthair kitten with feline autoimmune lymphoproliferative syndrome. Note the marked generalized lymphadenopathy and splenomegaly. **b** Histology of an enlarged lymph node reveals effacement of normal architecture (H&E ×40 magnification). The cells present within the lymph node uniformly react to antibodies against CD3 confirming a T-lymphocytic proliferation (*inset*)

of the unique and fixed variants in the two BSH cats was of high impact, specifically a base insertion, implying that it caused a major disruption to the protein. The variant was an insertion of an adenine at position 14,607,400 within exon 3 of the *FASL* gene (Fig. 2a) in chromosome F1. The adenine insertion (c.413_414insA) resulted in a frameshift and a predicted premature stop codon at position 176 of the 280 amino acid protein chain (p.Arg140Lysfs*37). This alteration was predicted to result in a truncated FASL protein that almost completely lacks the extracellular

Table 2 Chromosome number, 6.2 *Felis catus* genome assembly position, SNP effect, functional class of each variant, and name of the gene containing one of the 15 non-synonymous variants, homozygous and unique to the BSH cats

Chr	Position	SNP effect	Functional class	Gene name
A2	24066731	Moderate	Missense	*ERC2* (ELKS/RAB6-interacting/CAST family member 2)
A2	97266077	Moderate	Missense	*CALCR* (calcitonin receptor)
B4	40310844	Moderate	Missense	*VWF* (Willebrand factor precursor)
B4	62874803	Moderate	Missense	*C12orf71* (C12orf71 homolog)
D3	696861	Moderate	Missense	*FBRSL1* (fisbrosin 1- like-protein)
D4	71784955	Moderate	Missense	*FAM206A* (family with sequence similarity 206, member A)
E1	42247154	Moderate	Missense	*KRT32* (keratin 32)
E1	60670289	Moderate	Missense	*ENSFCAG00000024632*
F1	**14607400**	**High**	**Frame shift**	***FASL* (tumor necrosis factor ligand superfamily member 6)**
F1	18778054	Moderate	Missense	*BRINP2* (BMP/retinoic acid-inducible neural-specific protein 2)
F1	20855974	Moderate	Missense	*TDRD5* (tudor domain-containing protein 5)
X	81618437	Moderate	Missense	*ENSFCAG00000024052*
X	81684689	Moderate	Missense	*ENSFCAG00000009245*
X	83430878	Moderate	Missense	*SLC25A53* (solute carrier family 25 member 53)
X	86884747	Moderate	Missense	*ENSFCAG00000024792*

The single high impact variant is highlight in bold font

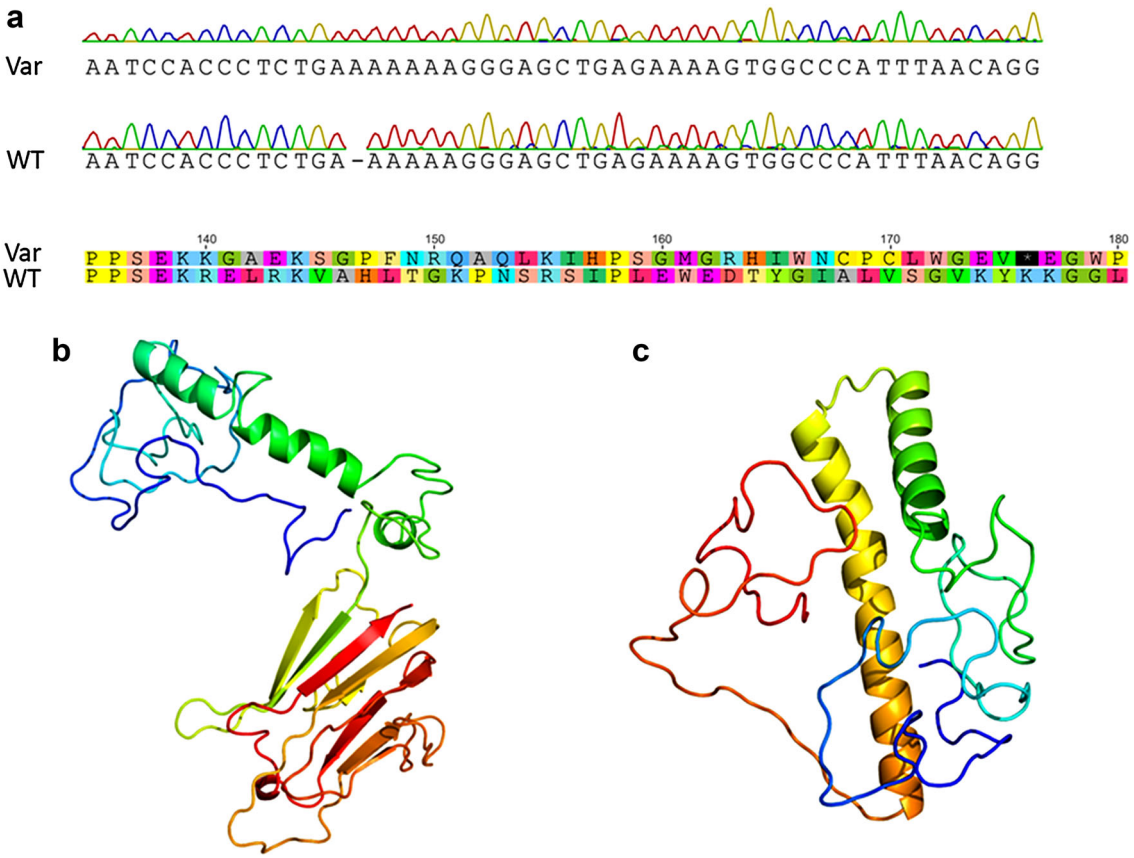

Fig. 2 **a** The genetic sequence of *FASL* in affected and wild-type British shorthair kittens. The insertion of an adenine (c.413_414insA) in the affected cats results in a stop codon in the predicted protein sequence. **b** and **c** The predicted effect of the identified variant on FASL protein structures (**b**) illustrates the predicted wild-type protein, while (**c**) is the truncated protein predicted in affected kittens

domain and is therefore unlikely to bind to the FAS receptor (Fig. 2b, c). *FASL* was considered a candidate gene for the disease and both affected cats were homozygous for this variant. The feline *FASL* gene is located on cat chromosome F1 from position 14,601,665 to position 14,609,054 of the *Felis catus 6.2* genome assembly (Montague et al. 2014), and like humans is predicted to have four coding exons (Takahashi et al. 1994).

DNA was amplified from the NZ cases using primers specific to the region of DNA containing the variant. These primers amplified DNA from three affected BSH kittens, two putative BSH carriers who were the sire or dam of multiple affected kittens and an unaffected cat. The results were as expected with the three affected cats being homozygous for the *FASL* variant, the two putative carrier cats being heterozygous, and the control cat showing a homozygous wild-type genotype.

Genotyping of DNA from the 14 BSH cats from the Australian cattery that were suspected by the breeder to be carriers revealed that nine cats were heterozygous for the *FASL* variant. The remaining five cats were homozygous wild type on sequencing (Table 3).

Pedigree analysis revealed that the affected and identified carrier BSH cats in New Zealand and Australia shared multiple (>5) common ancestors that could all be traced back to a single mating that occurred between 3 and 11 generations earlier. A high degree of consanguinity was present within the pedigree of these cats.

The newly identified *FASL* variant was not identified in 510 archived DNA samples from numerous non-BSH cat breeds and populations (Table 4).

Discussion

ALPS is an inherited disorder caused by a decreased ability to trigger lymphocyte apoptosis (Fisher et al. 1995). In a cell from a normal individual, FASL induces apoptosis of FAS-expressing cells by binding to membrane-bound FAS receptor resulting in caspase activation and subsequent cell apoptosis (Ju et al. 1999). ALPS can result from loss of normal function of any of the components of the FAS-mediated apoptosis pathway and one of the diagnostic criteria for ALPS in humans is the detection of a variant in the *FAS*, *FASL*, or *caspase 10* (*CASP10*) genes (Oliveira et al. 2010). By whole genome sequencing two affected individuals and comparing their sequences to 82 healthy individuals already in the sequencing database, over 3000 variants were unique to the affected individuals and concordant with the phenotype. Of these variants, only 15 were predicted to have an effect on protein products, and the highly damaging adenine insertion within *FASL* represented the only candidate mutation based on protein function and known role in disease development.

Table 3 *FASL* sequencing results within British shorthair cats

Phenotype	#	Genotype		
		InsA/InsA	WT/InsA	WT/WT
Affected	5	5	0	0
Unaffected	16	0	11	5
Total	21	5	11	5

Table 4 *FASL* genotypes in 510 cats including random bred cats and cats from 29 cat breeds

Breed/population	#	Genotype		
		G/G	G/GA	GA/GA
Abyssinian	29	29	0	0
American curl	2	2	0	0
American SH	2	2	0	0
Bengal	62	62	0	0
Birman	8	8	0	0
Bobcat	12	12	0	0
Burmese	21	21	0	0
Chartreaux	6	6	0	0
Cornish rex	8	8	0	0
Devon rex	7	7	0	0
Egyptian mau	34	34	0	0
Japanese bob	30	30	0	0
Korat	3	3	0	0
Laperm	48	48	0	0
Maine coon	10	10	0	0
Manx	14	14	0	0
Norwegian FC	10	10	0	0
Ocicat	19	19	0	0
Oriental SH	31	31	0	0
Persian	8	8	0	0
Pixiebob	14	14	0	0
Ragdoll	7	7	0	0
Random bred	71	71	0	0
Russian blue	5	5	0	0
Siamese	19	19	0	0
Siberian	5	5	0	0
Sphynx	7	7	0	0
Tonkinese	7	7	0	0
Turkish angora	5	5	0	0
Turkish van	6	6	0	0
Total	510	510	0	0

Genotyping confirmed that the *FASL* variant is associated with the disease phenotype in BSH cats in Australasia.

FASL features a topological domain (from amino acid 1 to 80), a transmembrane domain (from amino acid 81 to 102), and an extracellular domain (from amino acid 103 to 281) (http://www.uniprot.org/uniprot/P48023). The detected variant is predicted to be translated to a truncated FASL protein that almost completely lacks the extracellular domain (p.Arg140Lysfs*37). Therefore, while the ability of the truncated FASL to interact with the FAS receptor was not tested, the significant conformational changes predicted in the defective feline FASL protein suggest that the altered FASL protein is unlikely to retain its ability to activate the FAS receptor. It is also possible that due to the presence of a premature translation termination codon within the aberrant *FASL* RNA, nonsense-mediated mRNA decay may have been initiated to prevent the truncated RNA from being translated into a possibly harmful polypeptide (Holbrook et al. 2004). Regardless of the precise mechanism, the resultant inability to trigger apoptosis in activated lymphocytes would account for the massive lymphadenopathy observed in affected kittens. The identification of the variant in the *FASL* gene in affected kittens confirms that the disease in kittens is analogous to human ALPS and we propose the disease be called feline-ALPS (FALPS).

The presence of a variant within *FASL* in these cats suggests the disease fits the subclassification of ALPS-FASLG (formerly ALPS-type Ib) (Li et al. 2016). Less than 1 % of human ALPS cases are caused by defects in *FASL*. In humans, homozygous loss of *FASL* has been reported in four children, where the complete loss of functional FASL resulted in a severe clinical manifestation of ALPS with marked lymphadenopathy and abdominal distension observed at two to three months of age (Del-Rey et al. 2006; Magerus-Chatinet et al. 2013; Nabhani et al. 2014). Two patients with heterozygous variants in *FASL* have also been reported. The first was a 52-year-old who was initially diagnosed with systemic lupus erythematosus (Wu et al. 1996). This patient was considered to have abnormally marked lymphadenopathy during the course of the disease, although the patient did not fulfill the criteria for a diagnosis of ALPS and the effect of the heterozygous *FASL* defect in this patient could not be determined definitively. In contrast, the second patient with a heterozygous variant in *FASL* developed ALPS at a young age, possibly because the altered FASL protein interfered with the binding of the normal FASL protein to the FAS receptor (Bi et al. 2007). In the BSH cats, all cats tested that showed clinical signs of disease had two copies of the defective *FASL* gene. These cats showed severe onset of clinical signs at an early age consistent with the disease seen in people with homozygous *FASL* mutations. While there is currently no evidence that cats with a heterozygous variant in *FASL* show clinical disease, heterozygous carriers of *FASL* mutations identified in this study represent a valuable animal model to study whether heterozygous variants in *FASL* predispose to immune dysfunction or lymphoid neoplasia. Moreover, in the hypothesis of nonsense-mediated mRNA decay, the feline model can elucidate if haploinsufficiency is observed with this aberrant *FASL* gene model.

The current diagnostic criteria for ALPS in humans include two obligate criteria and two primary accessory criteria, one of which must be present before the diagnosis can be made (Oliveira et al. 2010). The first obligatory criterion is the presence of chronic non-neoplastic lymphadenopathy lasting greater than 6 months. As previously reported (Aberdein et al. 2015), all BSH kittens with homozygous *FASL* mutations developed massive non-neoplastic lymphadenopathy. One kitten subsequently died shortly after diagnosis, while the others were euthanatized on humane grounds between 1 and 8 weeks after diagnosis due to the development of weight loss and anorexia. While none of the kittens lived long enough to have lymphadenopathy for 6 months, the lymph nodes in affected kittens continued to increase in size over the course of the disease suggesting the lymphadenopathy was likely chronic (Aberdein et al. 2015). The second obligatory criterion for human ALPS is the presence of increased circulating CD3+ CD4− CD8− 'DNT' lymphocytes, with similar cells often also present in enlarged lymph nodes (Lim et al. 1998). As previously reported, appropriate lymph node samples were only available from one affected BSH kitten. However, testing revealed a marked increase in DNT-lymphocytes in multiple enlarged lymph nodes from this individual (Aberdein et al. 2015). One of the primary accessory criteria is a demonstration of defective lymphocyte apoptosis. No samples were available to evaluate lymphocyte apoptosis in affected animals, although it should be noted that, as the defect was in *FASL*, apoptosis induced by exogenous FASL would have been normal in these cats as it is in people with *FASL* defects (Bi et al. 2007; Del-Rey et al. 2006). Overall, the marked non-neoplastic lymphadenopathy, the increased DNT cells, and the identification of a *FASL* variant are consistent with FALPS meeting the diagnostic criteria for human ALPS cases.

A significant difference between the BSH cats and most human ALPS cases is the severity of the clinical signs resulting in massive lymphadenopathy and cytopenia leading to death or euthanasia in affected kittens. This is in contrast to the generally mild, readily treatable, and often self-resolving disease seen in most human ALPS cases. However, most human ALPS cases develop in people with autosomal dominant heterozygous variants in the *FAS* gene (Worth et al. 2006). The defect shows incomplete penetrance suggesting that the mild clinical signs visible in most

patients are due to retention of some FAS function due to the presence of the one normal *FAS* allele. The disease in BSH kittens may therefore be more comparable with the disease seen in people with homozygous mutations in genes of the FAS apoptosis pathway. Affected people present with massive lymphadenopathy that is present from birth with intensive therapy required to prevent severe clinical disease and death (Magerus-Chatinet et al. 2013; Nabhani et al. 2014; Rieux-Laucat et al. 2003; van der Burg et al. 2000). As previously reported, treatment using high-dose oral prednisolone was ineffective in affected kittens (Aberdein et al. 2015). It is possible that the disease in the kittens could have been stabilized using treatments that are used to treat autoimmune cytopenias in more severe human ALPS cases such as mycophenolate mofetil or splenectomy (Li et al. 2016).

The confirmation of an ALPS-type disease in cats makes them the third species recognized to develop lymphoproliferation by this mechanism. Mice represent the other animal species with ALPS-type disease. Two murine models of *FASL* defects have been studied in detail. FASL knockout mice show clinical signs similar to those observed in the BSH cats with marked lymph node enlargement observed at around 6 weeks of age leading to death of over 50 % of mice by 16 weeks of age. Mice with generalized lymphoproliferative disease (*gld*) have a point mutation within the C-terminal region of *FASL*. As in cats, clinical disease in *gld* mice is only seen in autosomal recessive homozygous animals. However, compared to cats, *gld* mice develop a milder form of disease that typically is not clinically detectible until 3–5 months of age, with affected mice having a mean survival time of 12 months (Roths et al. 1984). The difference between the *gld* mice and the *FASL* knockout mice suggests that *gld* mice produce FASL protein that retains some ability to interact with the FAS receptor. The striking clinical similarities between the *FASL* knockout mice and affected BSH kittens provide additional evidence that altered FASL produced in affected cats has lost all affinity for the FAS receptor.

Variants in *FASL* are rare in people and were not detected in any of 52 normal individuals tested (Wu et al. 1996). Supporting an uncommon variant, homozygous *FASL* mutations have only been seen in four children, all from consanguineous families (Del-Rey et al. 2006; Magerus-Chatinet et al. 2013; Nabhani et al. 2014). Likewise in cats, to the authors' knowledge this disease has only been reported in Australasian BSH cats, and the *FASL* variant was not detected in any of 510 samples from non-BSH cats. Pedigree analysis of BSH cats from Australasia reveals many of the cats were related. Due to the strict quarantine regulations of Australia and New Zealand, importation of any new cat breed lines is difficult, thus, most breeds likely have reduced diversity and increased consanguinity compared to the breed in North America or Europe. Now that the causative defect has been identified, a survey of BSH from other countries should be conducted to determine if the mutation was imported into Australasia or whether the mutation developed de novo, and is unique, to this region. The first cats with this disease were observed in Australia in the early 1990s (S. Foster, D. Church, unpublished data), suggesting that this condition may have arisen spontaneously in a single Australian breeding cattery. The development of a routine genetic test is currently in progress and will allow cats to be screened for the mutation, allowing eradication of the disease in pet BSH cats.

In conclusion, the lymphoproliferative disease described recently in young BSH kittens can now be confirmed to be analogous with ALPS-FASL. Disease in these cats has only been observed in homozygous individuals and there is currently no evidence that heterozygous individuals are predisposed to disease. FALPS has an early onset and a rapidly progressive clinical course similar to that observed in people with homozygous *FASL* mutations and *FASL* knockout mice.

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