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Immunohistochemical evaluation of the activation of hepatic progenitor cells and their niche in feline lymphocytic cholangitis

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Abstract

Objectives The aim of the study was to compare the hepatic progenitor cell niche in healthy feline livers and the liver tissue of cats with lymphocytic cholangitis.

Methods Immunohistochemical stainings for vimentin, laminin, beta (β)-catenin and Notch1 intracellular domain (NICD) were used on formalin-fixed liver biopsies from affected (n = 12) and unaffected cats (n = 2).

Results All immunohistochemical markers used were expressed in more cells, or more intensely, in the liver tissue of cats with lymphocytic cholangitis than in the liver tissue of unaffected cats.

Conclusions and relevance Enhanced expression of vimentin, laminin, cytoplasmic/nuclear β-catenin and NICD in liver biopsies from cats with lymphocytic cholangitis indicates that the hepatic progenitor cell (HPC) niche is remodelled and activated. HPCs might provide insights into new regenerative treatment options for lymphocytic cholangitis in cats in the future.

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Introduction

Lymphocytic cholangitis (LC) is a chronic disease in cats, which affects the biliary tree and progresses slowly. Early signs include nausea and vomiting, changes in appetite and weight loss.[1,2] Jaundice is often present later in the course of the disease.[1,2] Hypergammaglobulinaemia is the most consistent finding and blood analysis sometimes reveals elevated hepatic enzymes and bile acids.[2] The World Small Animal Veterinary Association liver standardisation group described histological hallmarks of the disease, including hepatic lesions characterised by aggregates of inflammatory cells in portal tracts, and in and around bile ducts.[3] A ductular reaction (DR), also known as bile duct proliferation, is often seen.[3,4] DR is defined as a proliferating and expanding progenitor cell compartment.[5]

Chronic inflammation in the bile ducts causes dilations and strictures, and might eventually lead to fibrosis and cirrhosis.[6,7] Bacterial and immune-mediated components have been suggested, but the definitive aetiology of LC is still unknown.[8–12] This limits treatment options, which currently include corticosteroids and hepatosupportive drugs, such as ursodeoxycholic acid.[13]

The injured liver is capable of a remarkable and unprecedented regeneration based on proliferation of all mature cell lines present in the liver.[14] Only when regeneration capacity proves inadequate will hepatic progenitor cells (HPCs) be activated.[5,14,15]

Bipotent HPCs can give rise to both hepatocytes and cholangiocytes.[16] Upon activation, HPCs will proliferate,

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migrate from the canal of Hering to the site of injury, and differentiate into cholangiocytes or hepatocytes, depending both on the disease and on concurrent changes in their microenvironment (HPC niche).[5,17,18] The HPC niche, both a histological location and a functional unit, is of importance in maintaining and regulating HPC behaviour, supporting self-renewal, and balancing quiescence, proliferation and differentiation in response to injury.[18,19]

Putative HPCs have been demonstrated immunohistochemically before, both in healthy feline livers and in the livers of cats suffering from cholangitis.[5,20] In healthy feline livers, Ijzer et al demonstrated the presence of resident progenitor cells in 'a small ductular structure just beyond the limits of the portal area'.[5] This 'suggests the presence of progenitor cells in the canal of Hering', as demonstrated with immunohistochemical (IHC) staining for cytokeratin-7 (CK7).[5] In diseased liver (from cats suffering from acute hepatitis, acute fulminant hepatitis, hepatic lipidosis, neutrophilic cholangitis and lymphoma), a DR was demonstrated with CK7.[5] Recommendations for future research included IHC staining with cytokeratin-19 (CK19).[5] CK19 was later applied to the livers of cats suffering from lymphocytic cholangitis and 15/20 samples stained positively, demonstrating a DR.[20]

It is likely that feline bile duct proliferation involves both cholangiocytes and HPCs.[17] To our knowledge, studies on feline HPCs and the HPC niche in feline LC are lacking. Therefore, the purpose of this study was to evaluate the activation of HPCs and the HPC niche in feline LC by comparing the liver tissue of patients with lymphocytic cholangitis with the liver tissue of unaffected cats. Commonly used IHC markers in other species, that is, vimentin, laminin, beta (β)-catenin and Notch1 intracellular domain (NICD), viz the active form of Notch1, are investigated because a single specific HPC marker has not been identified yet, and many HPC markers have a shared expression with cholangiocytes.[17,21,22]

The markers used for IHC were selected based on studies in other species. Vimentin expression has been shown in hepatic progenitor cells of rats, mice, humans and dogs.[21,23–25] It is a common mesenchymal marker and has been shown to indicate proliferative activity of cells and an undifferentiated state of HPCs.[25,26]

Remodelling of the extracellular matrix (ECM) and deposition of laminin have been shown to play an important role in HPC activation in hepatic injury in both rodents and human beings.[19,27] Furthermore, laminin was recently shown to promote cholangiocyte differentiation of bipotent cells in human liver disease.[28]

The Wnt/β-catenin signalling pathway has a central role in hepatic and bile duct development and regeneration, promoting gene activation, inhibiting apoptosis and increasing cellular proliferation.[29–31] Recently, it was also shown to be involved in homeostatic renewal of the liver.[32] It is believed to be involved in the proliferation, migration and differentiation of HPCs in mice, zebrafish, dogs and humans.[17,23,29] Furthermore, active Wnt guides cells to a biliary phenotype.[23] When Wnt is activated, it shifts from a membranous staining pattern to a more cytoplasmic/nuclear staining pattern.

Notch is involved in the proliferation, differentiation and apoptosis in all stages of organ development, including healthy and diseased livers.[33] Notch1 was shown to be activated in human primary sclerosing cholangitis, feline mammary tumours and in HPCs of dogs with lobular dissecting hepatitis.[23,34,35] The expression of the proteolytically processed, and therefore active, NICD has previously been used to measure the activation of Notch1 in cats.[34]

To date, no publications exist on the usage of these markers on healthy or diseased feline hepatic tissue. HPCs might provide new regenerative treatment options for LC in cats in the future.

Materials and methods

All liver biopsies from cats with LC were taken for diagnostic purposes only between 1998 and 2010. Samples of unaffected control livers were obtained from two cats used in unrelated liver research, approved by the animal experiments committee of Utrecht University, as required by Dutch legislation.

Case selection

Liver biopsies Cats diagnosed with LC, based on typical histopathological findings, clinical signs, and elevated levels of bile acids and/or activities of liver enzymes, were identified from the registration system used by the Department of Pathobiology.[3,20] Eight cats were identified. Four cats had second biopsies taken 1–9 months after the first biopsy to evaluate the efficacy of therapies.[13] In total, 12 liver samples of cats diagnosed with feline LC and multiple samples (2–4 samples per cat per IHC staining) of control cats were available for this study (see Table 1 for details). LC patient 3 was used as a negative control. Canine tissue samples were used as positive controls.[17,18,36]

All samples were fixed in 10% neutral buffered formalin and embedded in paraffin.

Histology

A routine haematoxylin and eosin staining was used as reported previously to diagnose the patients and determine the degree of inflammation.[20]

Immunohistochemistry

Staining protocols Tissue samples were routinely processed to produce slides with freshly cut sections (5 µm) and mounted on Polysine slides. Deparaffinisation and rehydration were performed in a series of xylene, alcohol and milli-Q baths (5 mins each). Antigen retrieval was obtained with Tris/EDTA buffer (TE; for β-catenin

Table 1 Characteristics of patients and biopsy materials

Identification	Sex	Age at diagnosis (years)	Breed	Therapy after first biopsy was taken	Biopsy type	Number of portal areas for evaluation
LC1 A	M*	14	DSH	–	Needle	>5
LC1 B	M*	14	DSH	P	Needle	0
LC2 A	M*	13	DSH	–	Needle	>5
LC2 B	M*	13	DSH	U	Needle	3
LC3 A	M*	9	NFC	–	Needle	5
LC3 B	M*	9	NFC	U	Wedge	>5
LC4 A	M*	9	DSH	–	Needle	4
LC4 B	M*	9	DSH	P	Wedge	>5
LC5	M*	13	DSH	–	Needle	>5
LC6	F*	14	DSH	–	Needle	>5
LC7	M*	12	DSH	–	Needle	3
LC8	F*	14	DSH	–	Needle	4
C1	M	1	DSH	–	Wedge	>5
C2	M	1	DSH	–	Wedge	>5

*Neutered
LC = cat with lymphocytic cholangitis; A = first biopsy; M = male; DSH = domestic shorthair; B = second biopsy; P = prednisolone; U = ursodeoxycholic acid; NFC = Norwegian Forest Cat; F = female; C = unaffected control

Table 2 Primary antibodies and antigen retrieval and washing buffer methods

	Source	Type	Clone	Company	Dilution	Antigen retrieval	Washing buffer
Laminin	Rabbit	Polyclonal		Abcam	1:100	PK	PBS and PBS/T
β-catenin	Rabbit	Polyclonal		Abcam	1:2500	TE, pH 9.0	PBS and PBS/T
Vimentin	Mouse	Monoclonal	RV203	Abcam	1:300	Citrate, pH 6.0	PBS and PBS/T
Notch1/NICD	Mouse	Monoclonal	mN1a	Merck/Millipore	1:200	TE, pH 9.0	TBS and TBS/T

PK = proteinase-K; PBS = phosphate-buffered saline; PBS/T = phosphate-buffered saline with Tween-20; TE = Tris/EDTA buffer; NICD = Notch1 intracellular domain; TBS = Tris-buffered saline; TBS/T = Tris-buffered saline with Tween-20

and Notch1/NICD), 10 mM hot citrate buffer (for vimentin), and proteinase-K (for laminin). TE and citrate antigen retrieval was performed by heat-induced epitope retrieval (98°C water bath for 30 mins, cooling down at room temperature [RT] for 30 mins). Proteinase-K ready-to-use (Dako), a proteolytic-induced epitope retrieval method, was incubated for 10 mins at RT. After rinsing in phosphate-buffered saline with 0.01% Tween-20, pH 7.4 (PBS/T; for β-catenin, vimentin and laminin) and Tris-buffered saline with 0.05% Tween-20, pH 7.6 (TBS/T; for Notch1/NICD) for 2 mins twice, endogenous peroxidase activity was blocked by incubating the slides with Dual Endogenous Enzyme Block (Dako) for 10 mins at RT. A second rinsing step with PBS/T or TBS/T was performed (5 mins, three times) and then background staining was reduced with 10% normal goat serum in PBS (for β-catenin, vimentin and laminin) and TBS (for Notch1/NICD) for 30 mins at RT. Primary antibodies were diluted based on earlier findings and optimised for use on feline liver samples (see Table 2 for details) in Antibody Diluent with background reducing components (Dako) and incubated in a humidified chamber at 4°C overnight. Before incubating secondary antibodies of the EnVision + System-horseradish peroxidase (HRP)-labelled polymer (Dako), slides were rinsed in PBS/T or TBS/T (5 mins, three times). Then, slides were incubated with secondary goat-antimouse (for Notch1/NICD and vimentin) or goat-antirabbit (for β-catenin and laminin) antibodies for 45 mins at RT and rinsed in PBS or TBS (5 mins, three times). Slides were incubated with freshly made diaminobenzidine (Dako) as substrate for HRP for 5 mins. After rinsing three times in milli-Q (5 mins each), slides were counterstained with haematoxylin quick-stain H-3404 (Vector Laboratories) for 10 s, whereupon the slides were placed under running tap water for 10 mins. Dehydration was performed by 60%, 70%, 80%, 96% (twice) and 100% alcohol baths and two xylene baths. Finally, slides were covered with Vectamount (Vector Laboratories).

Secondary antibodies against β-catenin and laminin act as mutual internal controls as they both are polyclonal IgG rabbit antibodies and have a distinct staining pattern. In the same way, the antibodies against NICD and vimentin, both being mouse monoclonal IgG1

Table 3 Summary of immunohistochemistry in cats with lymphocytic cholangitis (LC) and controls

	Portal area			Hepatic parenchyma	
	Lymphocytes	Cholangiocytes	ECM	Hepatocytes	Stellate cells
Vimentin					
Unaffected	– absent	–	+	–	+
LC	+++	+++	+++	–	+
Laminin					
Unaffected	– absent	–	+	–	–
LC	–	++/+++	+	+*	–
β-catenin					
Unaffected	– absent	++/+++ membrane	–	++/+++ membrane	–
LC	–	+++ cytoplasm/nucleus; ++/+++ membranes	–	++/+++ membrane; + cytoplasm†	–
NICD					
Unaffected	– absent	–	–	+ cytoplasm	–
LC	–	–/++	–	+ cytoplasm	–

*Non-specific background staining
†Not observed in all slides
ECM = extracellular matrix; NICD = Notch1 intracellular domain; (–) = no staining; (+) = weak staining; (++) = moderate staining; (+++) = strong staining

antibodies, presented as controls for a specific secondary antibody binding. Non-specific binding and background staining were evaluated by comparing the staining patterns for β-catenin and laminin, and NICD and vimentin, respectively. For the negative control, the primary antibody was omitted.

Staining (yes, no), intensity of positive staining (weak, moderate or strong) and location of immunoreactivity were evaluated for all markers used.

Results

Immunohistochemistry was performed on unaffected and diseased feline liver tissue due to LC, to determine the expression and location of vimentin, laminin, β-catenin and NICD. The results are summarised in Table 3. Positive and negative controls stained appropriately for all of the IHC markers.

Vimentin

In unaffected liver tissue, vimentin staining was weak in the ECM and vascular smooth muscle (Figure 1a). No staining was detected in the bile ducts in unaffected liver tissue. Occasionally, a stellate cell was seen in the hepatic parenchyma (Table 3). In feline LC, portal structures, including ECM and smooth muscle, stained strongly. Lymphocytes and bile ducts also stained strongly, and some individual cells in the DR also stained positively (Table 3, Figure 1b,c).

Laminin

The ECM stained weakly in unaffected liver tissue (Table 3, Figure 1d). No staining was detected in the bile ducts in unaffected liver tissue. In diseased liver tissue, a moderate-to-strong expression of laminin was seen in the cytoplasm of cells in the DR (Table 3, Figure 1e,f). A weak, non-specific background staining in the cytoplasm of hepatocytes appeared in some slides of cats with feline LC.

β-Catenin

In the unaffected liver samples, a moderate-to-strong staining of β-catenin in the membranes of hepatocytes and cholangiocytes was seen (Table 3, Figure 1g), indicating a low activation status of the Wnt/β-catenin signalling cascade. In the LC samples, a moderate-to-strong staining for β-catenin was seen in a membranous pattern in hepatocytes. In some slides, cytoplasm of hepatocytes stained moderately to strongly. Ductular structures in portal areas with cell infiltrates stained moderately to strongly in the cytoplasm/nucleus of the cells (Table 3, Figure 1h,i), while the membranes also exhibited moderate-to-strong staining.

NICD

In unaffected liver tissue (Table 3, Figure 1j) NICD staining was minimal. In diseased liver, NICD showed strong staining results in some cats (Table 3, Figure 1k,l). In other patients, the intensity varied from weak to moderate, with a propensity towards weak staining intensity. Expression was seen in the bile ducts as a staining around the nucleus of the cholangiocytes. A weak, non-specific cytoplasmic staining in hepatocytes was observed in all slides (controls and cats with LC).

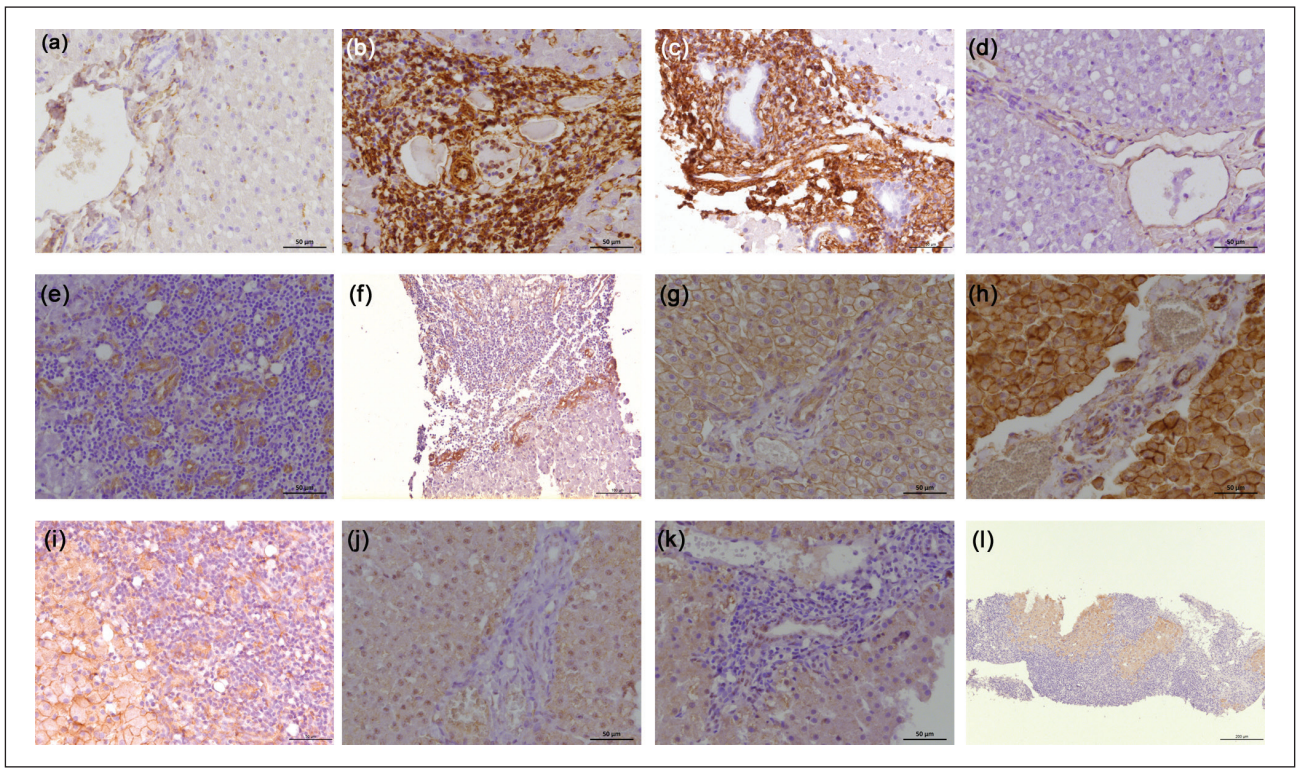

Figure 1 Immunolocalisation of the expression of hepatic progenitor cell markers in (a,d,g,j) unaffected and (b,c,e,f,h,i,k,l) liver tissue from cats with lymphocytic cholangitis (LC). (a) Weak vimentin expression in the extracellular matrix (ECM) and vascular smooth muscle of unaffected liver (C2), and (b [LC4]) and (c [LC5]) strong vimentin expression in portal structures in diseased liver. (d) Weak laminin expression in the ECM of (C2) unaffected liver and (e [LC6]) moderate to (f [LC2]) strong expression of laminin in the ductular reaction in diseased liver. (g) Moderate-to-strong expression of β-catenin in the membranes of hepatocytes and cholangiocytes in (C1) unaffected liver and (h [LC3]) and (i [LC6]) moderate-to-strong expression of β-catenin in membranes and cytoplasm/nucleus of hepatocytes and ductular structures in diseased liver. (j) Weak Notch1/Notch1 intracellular domain expression in (C1) unaffected liver and (k [LC5]) and (l [LC1]) weak-to-strong expression in the portal structures in diseased liver

Discussion

For this first and descriptive study of these immunohistochemical HPC markers on feline hepatic tissue, we used a small number of samples from cats with LC and unaffected cats. All markers in our study showed markedly enhanced expression in livers of cats with LC compared with unaffected liver samples. Additionally, the location of the expression differed between unaffected and LC liver tissue. This leads to our conclusion that the hepatic progenitor niche in cats with LC is remodelled and activated.

A limitation of our study is the small number of samples. This is a commonly encountered problem in many studies involving cats, although it is also encountered in many human liver research projects.[5,20,28,33,37] This made statistical analysis unreliable. Additionally, we included one sample with no portal tracts in our analysis. Although this sample could not contribute to our study for the stainings that are aimed at portal tracts, we still obtained information from it about the hepatocytes and the staining with β-catenin. Furthermore, this specific sample was taken after treatment with prednisolone, which made it interesting to include for further evaluation. Therefore, this is a descriptive study, and future research is needed with larger sample sizes, including various feline liver diseases, such as hepatic lipidosis, and neutrophilic cholangitis, in order to carry out statistical analyses.

Despite the small sample size, staining patterns were distinctly and consistently different in patients and unaffected cats. Although this is a much appreciated result, it made blinding of the authors for slide interpretation impossible.

Archival materials were used for patients. Fixation times and methods might have differed between these biopsies. Furthermore, unaffected samples were obtained from unrelated research, which may have influenced the way liver biopsies were taken and stored in that study. None of the samples from patients or unaffected controls were optimised for IHC research and this impacts the staining results obtained. Additionally, cats had been treated by their local veterinarians before being referred

to our referral clinic. Therefore, it was not possible to determine the chronicity of clinical disease. This may account for additional differences in staining results.

The control livers in this study expressed a low level of vimentin, suggesting the quiescent state of HPCs. In contrast, strong vimentin staining would indicate that cells are in an activated and proliferating state.[25,26] Vimentin was expressed in lymphocytes, bile ducts and individual cells in the DR in the portal areas of the LC livers. Although vimentin is traditionally regarded as a mesenchymal marker, it has been shown to be expressed in epithelial cells of humans and rodents.[26,38–41] More specifically, fibrosis of the hepatic tract has been attributed to cholangiocytes exhibiting mesenchymal characteristics, as demonstrated by the expression of vimentin.[40,41] As LC in cats may lead to fibrosis, our finding of vimentin-positive cells in the DR may indicate that the same processes described in humans and rodents take place in cats. This change in staining properties is attributed by some to epithelial–mesenchymal transition,[38,40] although other researchers have concluded that vimentin is sometimes needed as a structural scaffold for cells to build their structures on.[26,39,41]

The individual parenchymal cells in which vimentin was expressed were most probably stellate cells, as vimentin expression has previously been demonstrated in stellate cells.[20,42]

In this study, an expression of laminin in unaffected feline livers was seen in the ECM of portal areas, in and around vascular endothelium and bile ducts, and in sinusoidal endothelium adjacent to the portal area. This is similar to the results for healthy canine livers obtained in an immunofluorescence study.[18] In cats diagnosed with feline LC, an increase of laminin expression was seen in portal areas with many cell infiltrates, which is in accordance with earlier findings in cats and dogs.[18,20] This may indicate activation of HPCs in cats, as it does in humans and rodents.[19,27] Furthermore, the role of activated stellate cells in promoting biliary differentiation of HPCs has been demonstrated recently for rodents.[43] As LC is a biliary disorder, these findings correspond with our expectations.

The expression of β-catenin was evaluated because of its central role in the Wnt signalling pathway. In normal tissue, β-catenin is membrane-associated, but activation of the Wnt pathway leads to increased cytoplasmic/nuclear expression. The membranous expression of β-catenin in both hepatocytes and cholangiocytes in unaffected livers in the present study is in line with results from other mammals, indicating low activity of the Wnt signalling pathway in health.[23,44] In the LC samples, an increase in cytoplasmic/nuclear staining was seen in ductular structures, together with co-expression in the membrane. In feline tumours of the uterus and mammary glands, a simultaneous staining of cytoplasm and membrane was observed previously.[31,37] Thus, activated Wnt in cats may not shift completely from a membranous staining pattern to a cytoplasmic/nuclear staining pattern. Instead, the observed shift is more subtle as it includes a membranous staining pattern combined with a cytoplasmic/nuclear staining pattern. In our cats, the cytoplasm of cholangiocytes appeared to be darker than the cytoplasm of their hepatocytes. This is in line with the fact that Wnt/ β-catenin is involved in the development of bile ducts, the proliferation of HPCs and the promotion of a biliary phenotype. Furthermore, LC is not a liver disease, but a biliary disease.

NICD was expressed in several larger bile ducts in some LC samples; that is, wedge biopsies. As shown previously, differences between needle and wedge biopsies exist and may be attributed to a change in epitopes as a result of formalin fixation ('overfixation').[45] Fixation routines and times influence IHC staining results by protein cross-linking and masking of epitopes needed for IHC.[45]

Additionally, a weak cytoplasmic staining was seen in all hepatocytes. Boulter et al showed Notch1 to be highly expressed in biliary regeneration in mouse models.[43] Variable Notch/NICD expression is also described in two earlier studies on human primary sclerosing cholangitis (PSC).[33,35] Nijjar et al found no expression of Notch1 in five patients with PSC,[33] while Ishimura et al found Notch1 to be upregulated in the majority of patients with PSC (75% of 16 patients).[35] Our finding of variable upregulation of Notch1 in a series of similar size is consistent with findings in these two studies in humans with PSC.

Conclusions

Vimentin and laminin showed increased expression in portal areas of cats diagnosed with LC compared with unaffected feline livers. Additionally, β-catenin shifted from pure membranous to a more cytoplasmic/nuclear location. All markers showed enhanced expression in diseased livers, although NICD was not expressed in all patients.

These results indicate that the HPC niche is remodelled and activated in feline LC. Based on these and earlier discovered similarities between LC and PSC in humans, feline LC might prove to be a suitable translational model for human biliary disease. Furthermore, HPCs might provide new regenerative treatment options for LC in cats in the future.

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