LTβR signalling preferentially accelerates oncogenic AKT-initiated liver tumours

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ABSTRACT

Objectives The relative contributions of inflammatory signalling and sequential oncogenic dysregulation driving liver cancer pathogenesis remain incompletely understood. Lymphotoxin-β receptor (LTβR) signalling is critically involved in hepatitis and liver tumorigenesis. Therefore, we explored the interdependence of inflammatory lymphotoxin signalling and specific oncogenic pathways in the progression of hepatic cancer.

Design Pathologically distinct liver tumours were initiated by hydrodynamic transfection of oncogenic V-Akt Murine Thymoma Viral Oncogene Homolog 1 (AKT)/β-catenin or AKT/Notch expressing plasmids. To investigate the relationship of LTβR signalling and specific oncogenic pathways, LTβR antagonist (LTβR-Fc) or agonist (anti-LTβR) were administered post oncogene transfection. Initiated liver/tumours were investigated for changes in oncogene expression, tumour proliferation, progression, latency and pathology. Moreover, specific LTβR-mediated molecular events were investigated in human liver cancer cell lines and through transcriptional analyses of samples from patients with intrahepatic cholangiocarcinoma (ICC).

Results AKT/β-catenin-transfected livers displayed increased expression of LTβ and LTβR, with antagonism of LTβR signalling reducing tumour progression and enhancing survival. Conversely, enforced LTβR-activation of AKT/β-catenin-initiated tumours induced robust increases in proliferation and progression of hepatic tumour phenotypes in an AKT-dependent manner. LTβR-activation also rapidly accelerated ICC progression initiated by AKT/Notch, but not Notch alone. Moreover, LTβR-activated development coincides with increases of Notch, Hes1, c-MYC, pAKT and β-catenin. We further demonstrate LTβR signalling in human liver cancer cell lines to be a regulator of Notch, pAKT and β-catenin. Transcriptome analysis of samples from patients with ICC links increased LTβR network expression with poor patient survival, increased Notch1 expression and Notch and AKT/PI3K signalling.

Conclusions Our findings link LTβR and oncogenic AKT signalling in the development of ICC.

Significance of this study

What is already known on this subject?

▸ Lymphotoxin-β receptor (LTβR) inflammatory signalling is upregulated in patients with viral hepatitis and cholangitis and implicated in the initiation of hepatocellular carcinoma (HCC).

▸ Dysregulation of PI3K/AKT, β-catenin, and Notch pathways are frequently observed in HCC and intrahepatic cholangiocarcinoma (ICC).

▸ The interconnection between LTβR activation and oncogenic dysregulation towards the development of liver cancer remains incompletely understood.

What are the new findings?

▸ Oncogenic AKT cooperating with β-catenin upregulates LTβ/LTβR to facilitate liver tumour progression.

▸ LTβR agonism skewes AKT/β-catenin pathology towards a more ICC-like phenotype, as well as accelerates oncogenic AKT/Notch-initiated ICC formation in mice. LTβR-mediated tumour progression is dependent on oncogenic AKT and further accelerated when combined with catenin (CAT) or Notch signalling.

▸ LTβR is widely expressed and maintains oncogenic activity in human HCC and ICC cell lines. High levels of LTβR network expression correlates with increased AKT signalling, Notch1 expression as well as poor survival in patients with ICC.

How might it impact on clinical practice in the foreseeable future?

▸ Combination therapies are being extensively explored in preclinical and clinical studies for liver cancer treatment. Combining drugs targeting oncogenic AKT signalling, which have already been in development, with immune agents blocking the activity of the LTβR network may be a valuable new strategy.
and Notch are key oncogenic pathways that are frequently mutated or dysregulated in liver cancer. However, targeted therapies of these pathways so far have limited efficacy, in part due to our incomplete understanding of the relative contribution of inflammatory factors and their ability to collaborate with oncogenic pathways. Lymphotoxin $\beta$ receptor (LT$\beta$R) is a member of the tumour necrosis factor (TNF) superfamily of receptors, activated by the proinflammatory cytokines lymphotoxin (LT) $\beta$ heterotrimer and TNFSF14 (LIGHT). LT$\beta$ expression is primarily restricted to lymphocytes and is critical for lymph node formation and host defense. LT$\beta$R is expressed on most cells with highest expression on epithelial and myeloid lineages. LT$\beta$R signalling is broadly activated during chronic liver inflammation in patients with viral and non-viral hepatitis, cholangitis and HCC. LT$\beta$R signalling in mice has been shown to be critical for liver regeneration, and lipid homeostasis. It has also been demonstrated that aberrant expression of LT$\beta$ in hepatocytes is capable of inducing hepatitis and initiating HCC formation in mice through canonical nuclear factor $\kappa$B-light-chain-enhancer of activated B cells (NF-$\kappa$B)-dependent mechanisms. Collectively these studies establish a relationship between LT$\beta$R and HCC initiation; however the underlying oncogenic mechanisms driving LT$\beta$R-facilitated tumour progression remain incompletely understood. Our focus is on the role of LT$\beta$R signalling in modulating onogenesis using two pathologically distinct models of human liver cancer. Specifically, Sleeping Beauty (SB) mediated transposition, using plasmids containing oncogenic myristoylated-AKT (AKT)/$\Delta N$$\beta$-catenin (CAT) have been shown to initiate liver tumours consisting predominantly of hepatocellular adenoma with some regions of HCC, while AKT/Notch-intracellular domain (NICD) has been shown to selectively drive ICC. The novel potential roles of LT$\beta$R signalling in ICC were also evaluated in human cholangiocarcinoma cell lines and by comparisons with samples obtained from patients with liver cancer.

MATERIALS AND METHODS

Hydrodynamic transfection and Sleeping Beauty plasmids

Liver tumours were initiated in 7–8 week-old female C57/BL6 Jax mice by hydrodynamic tail vein injection of 10% volume/weight of 0.9% saline containing SB third generation (pT3) plasmids expressing oncogenes (transposon) and hyper SB transposase (HSB2) as previously described. Oncogenic AKT/Notch experiments were performed using a transposon:transposase ratio of 10:1 with concentrations of 14 $\mu$g/mL mouse myristoylated-AKT, 14 $\mu$g/mL human $\Delta N$$\beta$-catenin and 3.125 $\mu$g/mL HSB2 transposase with constructs obtained as described. Oncogenic AKT/Notch experiments were performed with 3.125 $\mu$g/mL AKT and 12.5 $\mu$g/mL mouse NICD and 1.6 $\mu$g/mL HSB2. cMET/CAT experiments were performed with 5.625 $\mu$g/mL human MET and 5.625 $\mu$g/mL human CAT. Single oncogene experiments were performed using equal amounts of transposon expressing plasmids and pT3 at a 10:1 (transposon:transposase) ratio.

Gaussia Luciferase assay

Cohydrodynamic transfection of 0.625 $\mu$g/mL pT3 containing Gaussia luciferase with oncogene plasmids was used to measure relative oncogene expression and tumour burden as described in Subleski et al (unpublished, manuscript under review). Serum was periodically assayed (1:400) with Dual-Luciferase Reporter Assay (#E1910; Promega) as per manufacturer’s instructions.

Lymphotoxin reagents

Mice were intraperitoneally injected with either 100 $\mu$g of agonistic antibody LT$\beta$R clone 4H8 (anti-LT$\beta$R), 0.1% normal rat serum,100 $\mu$g rat IgG (Ig, control), 100 $\mu$g of soluble decoy receptor, mouse LT$\beta$R-Fc (LT$\beta$R-Fc) produced in 293T cells and purified by affinity chromatography or 100 $\mu$g human IgG (IgG, control) in 200 $\mu$L twice per week for durations lasting 4 weeks or 8 weeks. Administration of reagents was initiated on day 10 post hydrodynamic transfection.

Mouse tissue processing

Mouse livers were either submerged in RNA later (Life Technologies, Gaithersburg, Maryland, USA) for RNA or snap frozen for protein lysate preparations. Livers were also fixed in 10% neutral-buffered formalin phosphate (Fisher Scientific, Pittsburgh, Pennsylvania, USA) to be embedded in paraffin or optimal cutting temperature compound (OCT).

Immunohistochemistry

The Histology and Tissue Core Facility at the Frederick National Laboratory for Cancer Research routinely performed H&E, Masson’s trichrome and immunohistochemical (IHC) staining for LT$\beta$R, LT$\beta$, $\alpha$-feto protein (AFP), CK19, CK8, Notch1, Hes1, pAKT, AKT, Glypican-3, c-MYC, Ki67, $\beta$-catenin and CD34 using paraffin sections, and Oil Red O staining with frozen sections. IHC antibodies and methods are listed in online supplementary material. H&E stained liver sections were used to histologically evaluate tumour phenotype/severity. Livers with distinct regions or nodules were counted, with significance determined using Mann-Whitney U test. Livers with less distinct, coalescing lesions were histologically scored with nodule/region distinguished by evidence of compressed margins. Lipogenic hepatic foci were scored as follows: 1 (1–10 nodules) 2 (11–20 nodules) 3 (21–30 nodules) 4 (30 nodules), hepatoblastoma/HCC-like 1 (1–2 nodules) 2 (3–4 nodules) 3 (5–6 nodules) 4 (>7 nodules), cholangiocyte proliferation/dysplasia 1 (1–3 nodules) 2 (4–7 nodules) 3 (8–11 nodules) 4 (>12 nodules) or CC-like 1 (1–3 nodules) 2 (4–7 nodules) 3 (8–11 nodules) 4 (>12 nodules).

Immunoblotting

Cell line and liver tissue extracts were lysed in the lysis buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 400 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1% NP40, protease inhibitor and phosphatase inhibitor and normalised before western blot analysis was performed using antibodies listed in online supplementary material.

Nanostring and PCR analysis

RNA extracted from livers and cells were subjected to reverse transcription and subsequently underwent quantitative PCR with the use of the Applied Biosystems Inc (ABI) 7300 real-time PCR system (Carlsbad, California). The following ABI primers (ABI identifier) were used: LT$\beta$R (Mm00440235_m1) and Gapdh (Mm99999915_g1). Analysis was performed according to ABI’s manufacturer’s protocol with target genes normalised to endogenous levels of GAPDH and 2$^{-}\Delta\Delta C(T)$ method used as previously described. Additional methods are listed in online supplementary material.

Hepatic cell lines and in vitro experiments

Human HCC cell lines HepG2, Huh1, HLE, Huh7 and human cholangiocellular cell lines Oz, KMBC, HuCCT1 and Mz-CHA-1 were generously provided by Dr Xin Wei Wang,
National Cancer Institute, Bethesda, Maryland. Additional methods are listed in online supplementary material.

Flow Cytometry

LTβR expression levels were determined using PE-labeled anti-LTβR (1:40) BD Pharmagen (Stamford, Connecticut, USA) with analysis performed by Becton Dickenson Canto cytometer. In vitro stimulation experiments were performed as indicated using agonistic goat antihuman LTβR.

Microarray analysis

The Llovet data set (ICC=143, normal biliary epithelial cells=6) was extracted from Geo Ominbus (GSE32225).19 The mean was calculated for each gene symbol and log 2-transformed using R script (V3.0.1). Log-transformed data were then imported into Biometric Research Branch (BRB) Array V4.3.2. Additional methods are listed in online supplementary material.

Statistical analysis

Differences between groups were compared using either Mann-Whitney U test or two-tailed unpaired Student’s t test. Survival differences were compared using Log-rank (Mantel-Cox) test. Statistical analysis was performed using GraphPad Prism 6.0 (La Jolla, California, USA). p values <0.05 were considered statistically significant.

RESULTS

LTβR signalling is critical for AKT/CAT-initiated hepatic tumour proliferation and progression

To investigate the role of the lymphotoxin signalling following oncogenic activation in the liver, C57BL6j mice were hydrodynamically transfected with the combination of AKT/CAT or empty vector (pT3). IHC staining for LTβR and LTβ of liver sections obtained 40 days after transfection of AKT/CAT showed increased levels of staining when compared with single oncogenes (figure 1A, B), with expression primarily restricted to regions of hepatic dysplasia. Isolated liver RNA obtained 40 days after AKT/CAT transfection displayed a modest increase in LTβ, while in contrast, LTβR ligand LIGHT (TNFSF14) and the LTα mRNA levels were not significantly changed relative to empty vector (pT3) control (see online supplementary figure S1A). Quantitative PCR revealed a mean 1.8-fold increase in LTβR in AKT/CAT-initiated tumours relative to pT3 transfected control livers (see online supplementary figure S1B). IHC staining for LTβR revealed increased expression by day 14 which intensified with the presence of tumour at day 49 and day 85 (see online supplementary figure S1C). LTβR ligand competitive antagonism was evaluated using a soluble form of the LTβ receptor (LTβR-Fc). LTβR-Fc was evaluated using a soluble form of the LTβ receptor (LTβR-Fc). LTβR-Fc was administered in a daily dose of 200 μg and/or oncogene integration,11 and continued for 8 weeks. LTβR-Fc significantly extended median survival to 206 days versus 157 days in Ig-treated control mice (figure 1C). A distinguishing feature of AKT/CAT-initiated oncogenesis is lipid accumulation,14 which is also associated with chronic LTβR activation.12 Treatment with LTβR-Fc decreased lipid accumulation as detected by Oil red O staining for neutral fats and quantitative microscopy (figure 1D). Moreover, prolonged administration of LTβR-Fc significantly reduced mean serum levels of the cotransfected oncogene reporter Gaussia luciferase by threefold (figure 1E) confirming a reduction in tumour burden. Quantitative analysis of liver IHC staining for pAKTser473, CAT and the proliferation marker Ki-67 demonstrated significantly reduced levels of oncogenes and the number of proliferating hepatocytes (figure 1F), but failed to significantly alter serum liver aspartate transaminase (AST)/alanine transaminase (ALT) enzyme and total bilirubin levels (see online supplementary figure S2A). There was no significant change in tumour morphology by Fc-treatment (see online supplementary figure S2B). AKT/CAT-transformation of hepatocytes and subsequent LTβR/LTβR upregulation are thus implicated in tumour proliferation and progression.

LTβR agonism enhances the proliferation, progression and prevalence of lipogenic and ICC-like tumours

Malignant transformation of hepatic adenoma to carcinoma is frequently accompanied by mutation of the β-catenin pathway.21 To investigate the ability of LTβR activation to drive AKT/CAT aggressiveness, we administered agonistic anti-LTβR mAb, clone 4H8 (anti-LTβR) for either 4 weeks or 8 weeks beginning at day 10 post transfection of C57BL6j mice. Survival was significantly reduced with chronic anti-LTβR treatment (figure 2A), and dramatically different tumour frequencies were observed between livers from control and anti-LTβR-treated mice at day 40 (figure 2B). A 13.3-fold increase in the serum level of cotransfected luciferase was detected in AKT/CAT/anti-LTβR mice (2.4×10^6 relative luciferase units, RLU) compared with the Ig control (1.8×10^5 RLU) at day 40 (figure 2C), which was consistent with the threefold increase in liver weights at day 40, (lg=1.01(g) vs anti-LTβR=3.4 g) (figure 2C). Additionally, serum liver enzymes ALT and AST were significantly elevated at day 40 following anti-LTβR treatment (figure 2C). Increased hepatocyte and cholangiocyte proliferation were also observed in AKT/CAT/anti-LTβR livers at day 40 as detected by IHC staining for the proliferation marker Ki-67 (figure 2D), with multiple coalescing proliferative regions of hepatic dysplasia with cellular atypia observed in hepatocellular and bile duct regions. Consistently, increased frequency of IHC staining was observed for AKT and CAT oncogenes that localised together and in areas of cellular dysplasia, reflecting increased tumour burden (figure 2D). Quantitative analysis of IHC confirmed significant increases in AKT and CAT, as well as Ki-67 expression in AKT/CAT/anti-LTβR livers (figure 2E). These data suggest that LTβR-activation accelerates AKT/CAT-initiated tumour formation and progression. It should be noted that in the absence of AKT/CAT oncogene expression, anti-LTβR failed to significantly alter proliferation, endogenous oncogene expression (see online supplementary figure S3A), and serum liver enzyme levels (see online supplementary figure S3B). We next focused our analysis on morphological characteristics of liver tumours harvested at day 40 or from moribund mice subsequent to chronic activation of LTβR. AKT/CAT-initiated tumours display multiple pathologies.18 Therefore, we further characterised nodules based on several morphological and molecular characteristics. Specifically, lipogenic hepatic foci are comprised largely of lipid fluid hepatocytes displaying clear cell morphology, as defined by with Oil red O ere(figure 3A) and AFP negative staining (see online supplementary figure S4). Hepatoblastoma/HCC-like nodules display a diverse morphological continuum of predominantly epithelial small cell, undifferentiated subtypes (figure 3B) with trabecular and cholangioblastic features including regions of desmoplasia (see online supplementary figure S5B arrow) which are present as tumours progress. These nodules stain positive for the hepatic stem/progenitor cell markers, Epithelial cell adhesion molecule (EpCAM) (figure 3B) and Glypican-3 with sporadic AFP staining (see online supplementary figure S4). Furthermore, CD34 a potential marker for detecting HCC/ICC cancer stem cells23.
and vascular endothelial cells,\(^{24}\) was also increased following LT\(\beta\)R triggering (see online supplementary figure S4). ICC-like lesions form diffuse, ductular/pseudoglandular patterns with the appearance of mitotic figures (figure 3C arrows) with ICC-like nodules staining positive for CK8 (a marker of preneoplastic hepatic lesions\(^{15}\) \(\text{25}\)), cholangiocyte marker CK19 (figure 3C), Ki-67, and Masson’s trichrome (fibrosis) (see online supplementary figure S4). Moreover, transposon expression, pAKT and \(\beta\)-cat IHC staining was observed in AKT/CAT-associated morphologies (see online supplementary figure S4). Histological evaluation of H&E stains from day 40 and moribund AKT/CAT-transfected livers following 4 weeks or 8 weeks of anti-LT\(\beta\)R or Ig treatment was performed (figure 3D, E). Agonist anti-LT\(\beta\)R treatment aggressively increased tumour burden (figure 2) with significant increases in lipogenic foci and ICC-like nodules observed at day 40 (figure 3D). Given the coalescing nature of the resulting tumours and sheer numbers of nodules in moribund livers, histological scoring was performed as described in Methods. Moribund livers following 8 weeks of anti-LT\(\beta\)R or Ig treatment displayed a preponderance of lipogenic foci with mean histological scores of 3.7 for anti-LT\(\beta\)R and 3.5 for Ig (figure 3E) and similar incidence of hepatoblastoma/HCC-like tumours following agonism (figure 3E). In contrast, anti-LT\(\beta\)R livers were interspersed with regions of ICC-like lesions, with mean histological scores of 0.35–3.0 in Ig and anti-LT\(\beta\)R livers, respectively. Furthermore, ICC mediator Notch1 and its downstream target Hes1\(^{26}\) were exclusively detected in AKT/CAT ICC-like lesions (figure 3F).

![Figure 1](https://example.com/figure1.png) **Figure 1** Upregulated LT\(\beta\)/LT\(\beta\)R expression is critical for AKT/CAT-initiated tumour progression. Immunohistochemical staining (IHC) of livers harvested day 40, post injection with pT3, AKT, CAT or AKT/CAT with antibodies against LT\(\beta\)R (A) and LT\(\beta\) (B). Arrows denote representative areas of positive staining. (C–F), LT\(\beta\)R-Fc (100 \(\mu\)g) or control IgG (100 \(\mu\)g) were administered twice/week in AKT/CAT-injected mice starting day 10, post oncogene injection, and continued for 8 weeks (C–E) or 4 weeks (F). (C) Survival curves were analysed and data is representative of two independent experiments (IgG and LT\(\beta\)R-Fc; \(n=9\)). (D) Representative Oil Red O stains of moribund livers with quantitation. (E) Tumour progression in moribund mice was analysed by measuring serum levels of cotransfected reporter Gaussia luciferase (relative luciferase units, RLU). (F) Quantification of IHC staining for pAKT, CAT and the proliferation marker Ki-67 in the livers day 40 post AKT/CAT injections. (D and F), positive cell numbers (counts) in at least 10 non-lapping fields (\(n=3–5\) mice/group) were determined using cell profiler quantitation software. Bars represent mean values ±SEM. *\(p<0.05\); **\(p<0.01\). Scale bars, 100 \(\mu\)m. CAT, catenin.
LTβR activation rapidly accelerates AKT/NICD-initiated ICC

Based on the results described above, a role for LTβR signalling in the progression of ICC was further investigated using a recently described ICC model driven by oncogenes AKT and active form of Notch, NICD.15 Hydrodynamic transfection of AKT/NICD, combined with chronic administration of anti-LTβR, dramatically increased liver weight (VC=1.1 g to anti-LTβR=2.2 g) and levels of cotransfected oncogenic reporter, Gaussia luciferase increased twofold (figure 4A). AST serum levels were significantly elevated at day 40 (figure 4B). Gross examination of these livers suggests LTβR-activation rapidly accelerates progression and pathogenesis of ICC (figure 4C). H&E staining of AKT/NICD ICC nodules suggests well defined foci with a ductular/pseudoglandular morphology (figure 4C) and the frequent appearance of mitotic figures (see online supplementary figure S6 arrows). IHC staining revealed increased expression of CK8, CK19, CD34 and Ki-67 in anti-LTβR treated livers (figure 4C). Likewise, anti-LTβR livers displayed increased staining of transfected Notch1, mediator Hes1 and oncogenic drivers NF-xB p65, pSTAT3 and c-MYC observed in ICC nodules (figure 4D). LTβR-accelerated tumour burden was further documented by increased levels of the transfected oncogenes NICD, with increased levels of AKT, activated pAKTThr308, pAKTSer473, NICD and Hes1 observed at day 40 (figure 4E) by western blot with liver lysates derived from AKT/NICD/anti-LTβR treated mice. To understand the mechanisms of LTβR-facilitated ICC progression, we next examined whether LTβR agonism indirectly activates other functionally validated liver cancer pathways.3 Increased levels of CAT, c-MYC, and pSTAT3Tyr705 were detected in AKT/NICD/anti-LTβR livers (figure 4E). Furthermore, cyclinD1 and E1, recently shown to
regulate ICC through interaction with p27,26–28 were elevated following LTβR activation (figure 4E). To corroborate AKT/NICD-related molecular findings, IHC staining was performed on AKT/CAT-initiated tumours, with NF-κB p65 selectively expressed in ICC-like lesions (see online supplementary figure S7A). Furthermore, in the AKT/CAT and AKT/NICD models, a significant increase in c-MYC transcription was observed following LTβR agonism (see online supplementary figure S7B).

LTβR agonism preferentially enhances AKT-initiated hepatic tumour development and reduces survival

We next investigated the ability of LTβR signalling to modulate hepatic tumour progression in models driven by only AKT,29 CAT,30 31 or NICD.26 Sequential serum analyses of Guassia Luciferase, AST, ALT and total bilirubin levels suggest that anti-LTβR treatments selectively promote AKT-initiated tumour progression (figure 5A), which was consistent with significantly increased liver weights at day 90 (figure 5B) and survival observed in AKT/anti-LTβR-treated mice (figure 5C). Day 90 histological evaluation of tumour nodules supports direct collaboration between AKT and LTβR signalling, averaging 11.4 (Ig, n=7) vs 29.4 (anti-LTβR, n=7) nodules/liver following 8 weeks of treatment. In contrast, tumour burden in CAT/anti-LTβR and NICD/anti-LTβR-treated mice was unchanged relative to Ig control (figure 5C, D). Similar to AKT/anti-LTβR, AKT and AKT/anti-LTβR tumours were predominantly lipogenic hepatic foci, with LTβR agonism inducing the appearance of ICC-like nodules/regions by day 90 (figure 5D) and increasing frequency in moribund mice. In these mice, the mean histological score increased from 1.0 (Ig, n=6) to 3.0 (anti-LTβR, n=5) (figure 5E). Transfection of CAT results in the formation of distinct hepatoblastoma and HCC-like nodules at day 90 and day 400 (figure 5D, E), while NICD transfected livers were similar in morphology to AKT/NICD-initiated livers; solely ICC at day 90 and time of morbidity (Fig 5D, E).

Figure 3  Effect of LTβR overactivation on spectrum of tumour types present in AKT/CAT-initiated tumours. (A–C) Representative H&E staining (×400 with insets ×100) of morphologies that define tumour types as observed in AKT/CAT and AKT/CAT/LTβR initiated livers. (A) Lipogenic hepatic foci were large lipid dense hepatocytes displaying clear cell morphology which stain positive for Oil Red O. (B) Hepatoblastoma/HCC-like nodules displaying multiple characteristics, most commonly small cell, undifferentiated subtypes staining positive for EpCAM. (C) CC-like nodules/regions that form ductular/pseudoglandular patterns with mitotic figures (arrows) and staining for CK19. (D–E) Livers with distinct nodules were counted (D) with histological scoring performed to assess severity for livers with coalescing lesions (E). Histological evaluation of H&E stains from day 40 (D) and moribund (E) AKT/CAT-transfected livers following 4 weeks (D) or 8 weeks (E) of anti-LTβR (n=10 (D40), n=11 (moribund)), LTβR-Fc (n=8 (D40)) or Ig (n=10 (D40), n=20 (moribund)) treatment was performed. Mann-Whitney U test was used to determine significance. *p<0.05, ****p<0.0001. (F) Representative images of IHC stained intrahepatic cholangiocarcinoma (ICC)-like structures from AKT/CAT/LTβR moribund livers was performed using antibodies against Notch1 and Hes1. CAT, catenin; HCC, hepatocellular carcinoma; IHC, immunohistochemical.
Cholangiocyte proliferation/dysplasia was defined by appearance of cholangiocyte proliferation, biliary dysplasia and/or bridging, with the lack of well defined glandular patterns. Noteworthy changes were observed in biliary proliferation (H&E) following AKT or AKT/CAT/anti-LTβR, but not observed in pT3 or CAT/anti-LTβR livers at day 40 following 4 weeks of treatment (see online supplementary figure S8A, arrows). Furthermore, histo- logical scoring of day 40 and moribund H&E stained liver sections suggests AKT/CAT/anti-LTβR mediated cholangiocyte dysplasia (see online supplementary figure S8B). Therefore, LTβR signalling preferentially enhances AKT-initiated progression with the concomitant activation with CAT further enhancing the appearance of ICC-like morphology.

**LTβR signalling is involved in human ICC pathogenesis**

To confirm the novel role of LTβR signalling in the pathogenesis of human liver cancers, particularly ICC, we first screened by flow cytometry several human liver tumour cell lines for LTβR expression. We found that LTβR was widely expressed by all four cholangiocarcinoma cell lines (Oz, KMBC, HuCCT1 and Mz-ChA-1) we tested, as well as by two HCC cell lines Huh1 and HLE (figure 6A). Knockdown of LTβR in Huh1 and Oz cells with targeting siRNA resulted in decreased protein expression/activation of pAKTser473, CAT, NICD and Hes1 levels at 48 h post transfection (figure 6B), suggesting that LTβR signalling may be important for maintaining the activity of these oncogenes in human liver cancer cells. Since recent human ICC transcriptome analysis revealed elevated LTBR expression in a molecularly defined proliferative subtype of patients with ICC,19 we then performed further transcriptome analysis on this cohort which revealed LTBR expression was correlated with expression of LTBR (R^2=0.2699) and NOTCH1 (R^2=0.5081) (figure 6C). Moreover, ingenuity pathway analysis of differentially expressed genes of ICC vs normal were enriched in Notch, phosphatase and tensin homolog (PTEN) and PI3K/AKT signalling pathways (figure 6C, right panel). In addition, hierarchical clustering of ‘proliferative class’ genes revealed a subset of significantly regulated ‘proliferative’ genes similarly clustering with LTBR, NOTCH1 and Hes1 (figure 6D). Furthermore, an ICC cohort of Thai patients obtained for study through the TIGER-LC consortium (Chaisaingmongkol et al, manuscript in preparation).

**Figure 4** LTβR rapidly accelerates AKT/NICD-initiated intrahepatic cholangiocarcinoma (ICC) development. (A–E) Mice were hydrodynamically injected with AKT/NICD to initiate ICC formation, and then treated with anti-LTβR or Ig for 4 weeks starting on day 10, post oncogene delivery. Livers and serum were analysed at day 40 post AKT/NICD injection. (A) Liver weights (g) (n=19/group), serum Guassia Luciferase (relative luciferase units, RLU) (n=24/group) (B) aspartate transaminase (AST)/alanine transaminase (ALT) (units/L) and total bilirubin levels (n=9–10/group) were measured. (C) Representative images comparing multifocal tumours, as well as H&E and IHC staining for CK8, CK19, Ki-67, CD34 was performed. (D) Representative IHC staining using antibodies against pAKT, Notch1, Hes1, pSTAT3, NFκBp65 and c-MYC. Scale bar, 100 μm. (E) Western blot (WB) analysis for indicated markers was performed with liver tissue lysates from three representative mice per group. All scale bars represent mean values±SEM. Mann-Whitney U test used to determine significance, *p<0.05 *****p<0.0001. CAT, catenin; NICD, Notch-intracellular domain; IHC, immunohistochemical.
stratified LTβR high (n=43) expression with significantly worse survival as compared with ICC cases with LTβR low (n=42) expression (Figure 6E). Subsequent analysis of tissue samples from human ICCs revealed varying morphological patterns with positive staining observed for LTβR, β-catenin (membrane and nuclear), pAKT and Hes1 (see online supplementary figure S9). LTβR and LTβ positive cells with ICC and leucocyte morphology (see online supplementary figure S9, arrows) were observed. Together, these results suggest a link between the LTβR pathway and functionally validated drivers of ICC that strongly associate with human ICC.

DISCUSSION
These results reveal the novel interplay between the LTβR inflammatory pathway and key oncogenes that drive liver malignancy, particularly lipogenic hepatic foci and ICC-like lesions. We provide evidence that AKT/CAT combined activation can mediate the upregulation of LTβR expression and further demonstrate LTβR signalling is a central activator during tumour development. Moreover, prolonged LTβR activation significantly enhanced proliferation, skewing AKT/CAT-induced tumour morphology towards the appearance of ICC-like lesions and accelerating AKT/NICD-initiated ICC. Intriguingly, LTβR-mediated tumour progression was largely dependent on oncogenic AKT signalling as LTβR agonism failed to alter survival in single oncogene CAT or NICD-initiated tumour models. The LTβR is broadly expressed in human liver cancer cell lines and contributes to maintaining AKT activation and the accumulation of NICD. Transcriptome profiling of ICC cohorts confirmed a significant relationship between LTβR, NOTCH and

Figure 5 LTβR agonism preferentially promotes AKT-initiated tumour development. (A–E) 100 μg of anti-LTβR or Ig were administered twice/week in single oncogene CAT, AKT or NCID injected mice, starting day 10 post oncogene injection and continued for 8 weeks. (A) Serum analysis of Guassia Luciferase (RLU), aspartate transaminase (AST)/alanine transaminase (ALT) (units/L) and total bilirubin (mg/dL) levels were measured at indicated time points. (B–C) Liver weights (g) at day 90 (B) and percent survival were determined (C). (D–E) Nodules were counted or histologically scored using H&E stained liver sections from (D) Day 90 from AKT/Ig (n=7) AKT/anti-LTβR (n=7), CAT/Ig (n=7) CAT/anti-LTβR (n=7) and NICD/Ig (n=7) NICD/anti-LTβR (n=7). (E) Mean histological scoring (1 mild to 4 severe) was performed from H&E stained moribund or Day 400 liver sections from AKT/Ig (n=6) AKT/anti-LTβR (n=6) and NICD/Ig (n=4) NICD anti-LTβR (n=3) with nodule counts performed from CAT/Ig (n=4) CAT/anti-LTβR (n=4) livers. All scale bars represent mean values±SEM. Mann-Whitney or log-rank tests were performed. *p<0.05, **p<0.01. CAT, catenin; NICD, Notch-intracellular domain.
AKT/PI3K signalling pathways. Further, poor survival of patients with ICC significantly correlated with higher LTBR network expression. Defining the mechanisms underlying LTBR and/or LTBR upregulation during malignancy has been elusive. Simonin et al. recently elucidated a HCV-mediated mechanism that directly regulates tumour-specific increases in LTBR, independent of the oncogenic driver, N-MYC. We demonstrate specific concomitant oncogenic activation upregulates LTBR and LTBR expression in vivo. Similarly, we detected increased LTBR expression in AKT/NICD and cMET/CAT-hydrodynamically transfected livers (see online supplementary figure S10A, B). It is conceivable, oncogenic activation is intrinsically regulating LTBR induction due to immense transcriptional amplification following transformation, or mediated from extrinsic factors in the tumour milieu.

It is well established that liver cancer emerges following many years of chronic liver damage and compensatory hepatic cell proliferation. Interestingly, combined AKT/CAT or AKT/NICD activation was required for robust LTBR-mediated proliferation since hydrodynamic transfection of CAT or NICD alone failed to markedly enhance proliferation. We speculate the LTBR-mediated enhancement of proliferation is augmenting progression and skewing the pathological appearance of AKT/CAT-associated tumour phenotypes, such as ICC-like lesions. Based on work from Sia et al. who molecularly defined ‘inflammatory’ and ‘proliferative’ subclasses of human ICC tumours with higher LTBR expression (2.3-fold) associating with ‘proliferative’ ICC subclass and worse survival, it is reasonable to conclude LTBR or LTBR expression could serve as a marker for proliferating ICC. The appearance of cholangiocellular lesions in AKT-transfected livers has been described. We expanded on this observation and demonstrate that LTBR agonism + AKT and to a greater extent LTBR agonism + AKT/CAT, results in tumour pathology characterised by lipogenic hepatic foci with interspersed regions of ICC.
Recent studies have defined Notch1 as an essential regulator of liver progenitor cell fate and critical for the development of ICC. Moreover, IHC staining for AKT, Notch and CAT suggests ICC-like regions have elevated expression levels compared with adjacent hepatic foci. It is plausible that upregulation by either transposon or endogenous AKT, Notch and CAT oncogene in cholangiocytosis or liver progenitor cells initiates biliary tumour formation or hepatocyte dedifferentiation following malignant transformation as recently described. Furthermore, we provide evidence that LTβR signalling is also important in maintaining endogenous Notch1-ICD/Hes1 in human liver cancer cell lines and correlates with Notch1 expression/signalling in patients with ICC. Liu et al demonstrated that TNF, another TNF-superfamily member and subsequent IKK accumulation in HCC cell lines were capable of driving proliferative advantage via Notch1-FOXO1a suppression. Additionally, evidence identifies TNF-mediated Notch signalling in the progression of pancreatic cancer. Canonical NFκB signalling has been reported to be critical for AKT oncogenicity. Therefore, it is possible that LTβR-NFκB regulation of AKT with subsequent activation of NICD could facilitate tumour formation. Using the AKT/NICD ICC model, we demonstrate additional LTβR-specific molecular changes occur in association with upregulation of c-MYC, cyclin D1, cyclin E1 and CAT. It remains uncertain if these oncogenic proteins are directly regulated by LTβR signalling or indirectly induced by factors in the microenvironment. Regardless, accumulations of these well-established oncogenic drivers are capable of driving tumour development.

Two emerging hallmarks of cancer are deregulated metabolism and chronic inflammation. AKT/CAT-initiated livers are lipid dense, similar to those resulting from overactivated AKT which induces metabolic dysregulation, including a hypoglycaemic, hypoinsulinaemic and hypertriglyceridaemic phenotype with fatty liver and hepatomegaly. Moreover, hepatocyte LTβR signalling has also been reported to regulate metabolic function, lipid homeostasis, and recently the transition from non-alcoholic steatohepatitis to initiated HCC. It is therefore intriguing to speculate that the reduction in AKT/CAT-initiated tumour development by LTβR-Fc is mediated in part through a normalisation of metabolic functions. Chronic inflammation is an established risk factor and pathological marker for biliary tract cancers. LTβR-mediated oncogene activation failed to significantly enhance proliferation in human tumour lines, indicating the robust hepatocyte and cholangiocyte cell proliferation observed following LTβR agonism could in part result from increased microenvironmental factors. LTβR signalling is well known to recruit lymphoid and myeloid cells into lymphoid organs and tumours, and activation mediates accumulation of macrophages and NKT/T cells in association with increased expression of CXCL10 and CCL2 in AKT/CAT and AKT/NICD tumours (online supplementary figure 11 A, B). CXCL10 is expressed by hepatocytes during chronic viral hepatitis, induced by LTβR activation via NF-κB and is considered as one of the main chemoattractants for tumour-infiltrating immune cells. Furthermore, Dubois-Pot-Schneider et al provide molecular evidence that TNF, IL-6 and TGF-β-related signatures are increased during dedifferentiation of tumour-derived hepatocyte-like cells to progenitor cells. It remains unclear if LTβR mediated chemokine recruitment of inflammatory cells, and subsequent activation of NF-κB p65 and pSTAT3 observed in livers from AKT/CAT and AKT/NICD-transfected mice is promoting hepatocyte dedifferentiation or ICC-like formation. However, given the inflammatory aetiology of cholangiocarcinoma, the investigation of inflammation mediated through the LTβR, and its possible collaboration with molecular events to alter cell fate in the liver, may prove a rich avenue for further study. Collectively our results linking LTβR signalling and oncogenic activation suggest that drugs targeting LTβR signalling combined with AKT or Notch inhibitors may have important clinical implications.

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Contributors AJS and QJ contributed equally to the work. AJS and QJ conceived and designed experiments. AJS, QJ, TB, HD, DH and CH carried out experiments, interpretation of data and/or provided critical intellectual content. JMW, JS, IKS, IO and RHW discussed and interpreted data providing critical intellectual content. AJS and QJ wrote the manuscript. JMW, IO, CFW, XWW and RHW revised the final manuscript critically for important intellectual content. PSN and CFW provided critical reagents and contributed to planning animal experimentation. JC, SR and MR coordinated collection of human specimens. HD and XWW acquired data analysis and interpretation of results from human specimens.

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Competing Interests None declared.

Ethics approval All mice were handled, fed, housed and euthanised in accordance with an approved Frederick National Laboratory Institutional Animal Care and Use protocol.

Provenance and peer review Not commissioned; externally peer reviewed.

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REFERENCES


Supplemental Methods

Mice

C57BL/6 mice were originally purchased from either The Jackson Laboratories (Jax) (Bar Harbor, MA) or Charles River (NcR) (Frederick, MD) and were bred and maintained in specific pathogen-free (SPF) housing. Experiments were performed with appropriate littermate or strain and age-matched mice. Mice were humanely euthanized when moribund in accordance with Frederick National Laboratory Institutional Animal Use and Care protocol.

Serum assays

The Histology and Tissue Core Facility at the Frederick National Laboratory for Cancer Research routinely performed serum enzyme assays for Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and total bilirubin.

Immunohistochemistry

Immunohistochemical staining for LTβR, LTβ, AFP, CK19, CK8, Notch1, Hes1, pAKT, Glypican-3, and c-MYC was performed on paraffin sections using the Ultravision LP detection system (Thermo Fisher Scientific, Fremont, CA), according to the manufacturer’s instructions. Quantitation of positive nuclei or cells was performed using optimized cell profiler (Cambridge, MA) pipelines from at least 11 non-overlapping fields (n=3-6 mice). ICC tissue microarray (LV642) was purchased from US biomax, Inc. (Rockville, MD) and stained with antibodies against LTβ, LTβR, pAKT, Hes-1, and β-catenin.
### Immunohistochemical Antibodies

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### Western Blot antibodies

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Hepatic cell lines and in vitro experiments

Cells were transfected for 72h with 50nM of LTβR siRNA (cat# SR302740) or scrambled negative control siRNA (SR30004) obtained from Origene (Rockville, MD), using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. LTβR stimulations were performed using agonistic anti-LTβR 4H8 at 2ug/mL for indicated durations.

Nanostring and PCR Analysis

Reverse Transcription-PCR was performed using the following human primers β-actin fwd 5'-AGAGCTACGAGCTGCCTGAC-3', β-actin rev 5'-AGCACTGTGTGTTGGCGTACAG-3' and LTβR primers from Bioneer (cat# P327477). GX mouse inflammatory kit was used with 20ng RNA/sample as per NanoString Technologies (Seattle, WA) instructions and normalized to internal controls with analyses performed using nsolver software (Seattle, WA). Total mRNA counts/20ng is indicated as “counts.”
**Microarray analysis**

A class comparison was performed to investigate differential gene expression between ICC vs. normal control. Changes in expression of genes at the p<0.001 level were considered to be statistically significant. Ingenuity Pathway Analysis (IPA) of the significant genes was performed to test for enriched signaling pathways. For Pearson correlation analysis of genes vs. LTBR gene expression, log 2 data of the mean for each gene from ICC samples was extracted and graphed in Graphpad Prism 5. Statistically significant changes in gene expression between ICC vs. Normal biliary epithelial cells (494 genes of 1,394 “proliferative class genes” from Llovet data set passed the p<0.01 test). Amongst the 494 genes are Notch1, Hes1 and LTBR. Hierarchal clustering (pearson correlation-complete linkage were performed with the three genes (Genesis v1.7.6, ). (1)


**Supplemental Figure 1. Expression of LTβR and its ligands in AKT/CAT-injected livers.** A. mRNA expression (nanostring array) analysis of LTβR ligands was performed with RNA extracted from the livers day 40, post AKT/CAT injection. Total mRNA counts/20ng is indicated as “counts” B. Quantitative PCR (qPCR) analysis of LTβR mRNA levels from pT3 or AKT/CAT moribund livers. C. IHC to detect LTβR with AKT/CAT liver sections at day 14, 49 and 85. All scale bars depict mean values ±SEM. ns= not significant. Scale bars, 100 µm.
Supplemental Figure 2. LTβR-Fc treatment in AKT/CAT transfected mice failed to alter serum enzyme levels or lipogenic tumor morphology. Day 40 serum liver enzymes levels were measured from mice hydrodynamically transfected with AKT/CAT and treated with LTβR-Fc for 4 wks. B. Representative H&E staining from AKT/CAT moribund livers treated with LTβR-Fc.

Supplemental Figure 3. Sustained LTβR agonism alone is insufficient to drive hepatocyte proliferation. A. H&E and IHC staining for Ki-67, pAKT, and CAT with livers harvested at day 40 from mice hydrodynamically injected with pT3 empty plasmid and chronically administered anti-LTβR or Ig control for 4 weeks. B. Serum liver enzyme levels were measured at days 3, 10 and 30, post pT3 transfection following Ig or anti-LTβR treatment.

Supplemental Figure 4. Immunohistochemical staining characterizing AKT/CAT tumor-associated morphology. IHC staining was performed on liver sections from moribund AKT/CAT tumors using antibodies against α-feto protein (AFP), Glypican-3, CD34, Ki-67, cytokeratin (CK) 8, β-cat and pAKT. Lipid (Oil red O) and fibrosis (masson trichrome) staining was also performed.

Supplemental Figure 5. Evidence of cholangioblastic features in AKT/CAT hepatoblastomas. A. Representative H&E staining of AKT/CAT initiated tumors with cholangioblastic features and B. regions of desmoplasia (arrows).

Supplemental Figure 6. AKT/NICD induced ICC morphology. Representative H&E stained images form AKT/NICD tumor. Nodules form well defined ductular/pseudoglandular patterns with frequent appearance of mitotic figures (arrows).
Supplemental Figure 7. Expression of NF-κB p65 and c-MYC in AKT/CAT.  A. Representative images of IHC staining using antibodies against NF-κB p65 at day 40 and moribund.  B. c-MYC expression was measured from RNA isolated from AKT/CAT and AKT/NICD livers at day 40.

Supplemental Figure 8. Cholangiocyte proliferation/dysplasia selectively increased following AKT and LTβR activation. Cholangiocyte proliferation/dysplasia was defined by appearance of cholangiocyte proliferation, biliary dysplasia, bridging, however the lack of glandular patterns with histological scoring based on severity 1 (1-3) 2 (4-7) 3 (8-11) 4(>12).  A. Representative H&E images from pT3, CAT, AKT, and AKT/CAT transfected mice administered anti-LTβR for 4 weeks.  B. Histological scoring of H&E stained liver sections from day 40 and moribund AKT/CAT transfected livers.

Supplemental Figure 9. Immunohistochemical staining of human ICC. Representative IHC staining of ICC associated morphologies using antibodies against LTβR, LTβ, β-cat, pAKT, and Hes1 in five human ICC’s. Arrows depict LTβ positive cells with leucocyte morphology.

Supplemental Figure 10. LTβ expression in AKT/NICD and cMET/CAT initiated hepatic tumors. A-B. mRNA expression (counts/20ng) of LTβR ligands was performed from liver RNA day 40 post pT3 empty vector, AKT/NICD or cMET/CAT transfection.

Supplemental Figure 11. LTβR signaling increases tumor CXCL10, CCL2 and immune infiltration. A. Day 40 quantitation of immune marker IHC (F4/80, CD3, B220) staining from pT3 or AKT/CAT-transfected mice following anti-LTβR or Ig
treatment was performed from at least 12 non-overlapping fields, n=3-4/group.  B. Chemokine analysis of mRNA isolated from pT3, AKT/CAT or AKT/NICD transfected livers following treatment with anti-LTβR or Ig. n=3 or 4/group. All scale bars represent mean values ±SEM. Student T-test or Mann Whitney U was performed. *p<0.05, **p<0.01 *** and ****p<0.001.
A

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B

Days post pT3 transfection

- Ig
- anti-LTβR

Graphs showing AST and ALT levels over time.
Lipogenic hepatic foci

Hepatoblastoma/HCC-like

ICC-like
Mitotic spindles

AKT/NICD
**A**

**AKT/CAT/anti-LTβR**

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**B**

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**Counts**

- **pT3**
- AKT/CAT
- AKT/NICD

**Significance**

- ********
- *****

**Myc**

- *****
- ***
A

- pT3+ anti-LTβR
- CAT+ anti-LTβR
- AKT+ anti-LTβR
- AKT/CAT+ anti-LTβR

Day 40

B

Cholangiocyte proliferation/dysplasia

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- histological score

- Ig
- anti-LTβR