



MOLECULAR MECHANISMS OF TUMOR INITIATION IN BILIARY TRACT CANCERS

Mouse models of carcinogenesis and spatial
transcriptomics of human cancer

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Abbreviations

APC, Adenomatous Polyposis Coli ; Alb, Albumin ; AKT, V-Akt murine thymoma viral oncogene homolog ; APOBEC3B, apolipoprotein B mRNA editing enzyme catalytic subunit 3B ; ARID1A/B-ARID2, AT-rich interaction domain 1A/B-2; BAP1, BRCA1 associated protein 1; BCL2, B-Cell CLL/Lymphoma 2 ; BilIN, biliary intraepithelial neoplasia ; BRAF, V-Raf murine sarcoma viral oncogene homolog B ; BRCA1-BRCA2, breast cancer type 1/2 susceptibility protein; CAF, cancer associated fibroblasts; CCA, cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma ; pCCA, perihilar cholangiocarcinoma ; dCCA, distal cholangiocarcinoma; eCCA, extrahepatic cholangiocarcinoma ; CDK1-CDK4-CDK6, cyclin dependent kinase 1- 4-6 ; CK7, cytokeratine 7 ; CK19, cytokeratine 19 ; C-MYC, avian myelocytomatosis viral oncogene homolog ; COL1A1, collagen 1A1 ; Ct, threshold cycle ; CXCL5, C-X-C motif chemokine ligand 5 ; Cyclic AMP, cyclic adenosine monophosphate ;

DDC, 3,5- diethoxycarbonyl- 1,4 dihydrocollidine ; EGF, epidermal growth factor ; EGFR, epidermal growth factor receptor, synonym ERBB1 ; ELF3, E74 like ETS transcription factor 3 ; EIF2, eukaryotic translation initiation factor 2; EMT, epithelial mesenchymal transition; EPHA2: ephrin type-A receptor 2; ERBB2-3-4, V-Erb-B2 avian erythroblastic leukemia viral oncogene homolog 2-3-4; ERK, extracellular- regulated kinase; FGFR2, fibroblast growth factor receptor 2; GBC, gallbladder cancer; GNAS, guanine nucleotide binding protein (G Protein), alpha stimulating; GSEA, gene set enrichment analysis; HB-EGF, Heparin binding EGF like growth factor; HBV, hepatitis B virus; HES-1, Hairy And Enhancer Of Split 1; HRAS, Harvey rat sarcoma viral oncogene homolog;

ICPN, intracholecystic papillary neoplasm (gallbladder); IPMN, intraductal papillary mucinous neoplasm (pancreas); IPNB, intraductal papillary neoplasm of the bile duct; ITPN, intraductal tubulo-papillary neoplasm (pancreas); IDH1-IDH2, isocitrate dehydrogenase 1-2; IL-6, interleukin 6; IL-33, interleukin-33; JAK, Janus Kinase; K19, keratin 19; KRAS, V-Ki-Ras2 Kirsten rat sarcoma 2 viral oncogene homolog; LXR β , oxysterol receptor liver X;

MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia sequence 1 (BCL2-Related); MCN, mucinous cystic neoplasm; MDM2, double minute 2, human homolog of P53-binding protein; MDR1, multidrug resistance protein 1; MLL2-MLL3, myeloid/lymphoid or mixed-lineage leukemia 2-3; MUC1, mucin 1 (synonym: EMA: epithelial membrane antigen); MUC5AC/MUC6/MUC2, mucin5AC/mucin6/mucin2; MYC, avian myelocytomatosis viral oncogene homolog; NBL1, neuroblastoma 1, DAN family BMP antagonist; NRAS, neuroblastoma RAS viral (V-Ras) oncogene homolog; NR2F1, nuclear receptor subfamily 2 group F;

NRG1, neuregulin 1; Opn, osteopontin; PCA, principal component analysis; PD1, programmed cell death 1; PDL1, programmed cell death 1 ligand; PIK3, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PRKACA and PRKACB, protein kinase cAMP-activated catalytic subunit alpha/ beta; PSC, primary sclerosing cholangitis; (p)SMAD2-SMAD4, (phosphorylated) mothers against decapentaplegic homolog 2-4; (p)-STAT3, (phosphorylated)-signal transducer and activator of transcription 3;

RNF43, ring finger protein 43; RT-PCR, real time-polymerase chain reaction; RUNX3, runt-related transcription factor 3, synonym: AML2 acute myeloid leukemia gene 2; SCENIC, single cell regulatory network inference and clustering; SEMA3B-3F-4A, semaphorin 3B-3F-4A; SHH, Sonic Hedgehog signaling molecule; SOS2, son of sevenless homolog 2; SMO, smoothened, frizzled class receptor; SOCS3, suppressor of cytokine signaling 3; SOX17, SRY-Box transcription factor 17; SRC, V-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene; TNS4, tensin 4 (synonym: C-TEN); TGF β , transforming growth factor beta; TGF β R2, transforming growth factor beta receptor 2; TERT, telomerase reverse transcriptase; TP53, tumor protein P53; WIF1, Wnt inhibitory factor 1; YAP, Yes1 associated transcriptional regulator; eYFP, enhanced yellow fluorescent protein; YEATS4, yeats domain containing protein 4; ZIC1-4, Zinc finger protein family member 1-4; ZNF100, Zinc finger protein 100; ZNF717, Zinc finger protein 717.

I. Introduction

Biliary tract cancers constitute a heterogeneous group of aggressive tumors emerging in the biliary tree. They include gallbladder cancers (GBC) and cholangiocarcinomas (CCA), the latter being further subclassified into intrahepatic, perihilar and distal CCA according to their anatomical location along the biliary tract. Intrahepatic cholangiocarcinomas (iCCA) are located in the liver proximally to the right and left hepatic ducts and include large duct- and small duct-types. Perihilar cholangiocarcinomas (pCCA) affect the right, the left or the common hepatic duct located in the hepatic hilum, whereas distal cholangiocarcinomas (dCCA) involve the common bile duct (Figure 1). Perihilar and distal CCA are occasionally lumped together as extrahepatic CCA (eCCA) (1). Gallbladder cancers include cancers derived from the gallbladder and the cystic duct.

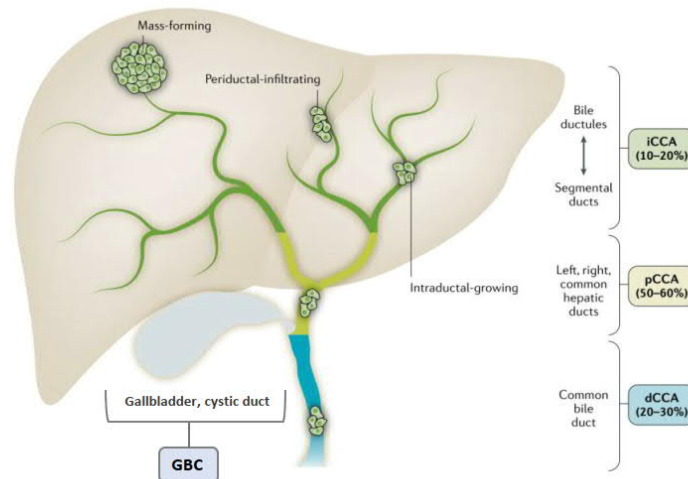


Figure 1 : Anatomical classification of biliary tract cancers. Biliary tract cancers include CCA and GBC. CCA are subclassified into iCCA, pCCA and dCCA. iCCA arise in the liver upstream to the left and the right hepatic ducts and present with distinct macroscopic growth patterns: mass-forming, periductal infiltrating and intraductal-growing. pCCA are located at the liver hilum and concern the left, the right and/or the common hepatic ducts. dCCA are located along the common bile duct. GBC derive from the gallbladder or the cystic duct. Adapted from Banales et al. Nat Rev Gastroenterol Hepatol 2020;17:557-588.

Biliary tract cancers are rare cancers worldwide and the incidence of CCA has increased globally during the last decades. The prognosis of biliary tract cancers remains dismal with a 5-year survival rate of 5%. This high mortality rate results in part from the fact that biliary tract cancers are asymptomatic at early stages, leading to diagnosis at an advanced stage, i.e. when curative treatment can no longer be considered. To improve patient outcome, there is a need for accurate screening methods allowing early detection of the disease. However, this requires a better understanding of the molecular events driving the development of these cancers at early steps. Therefore, unraveling molecular mechanisms of tumor initiation in biliary tract cancers is the goal of the present work.

I.1 Pathology of biliary tract cancers and their precursor lesions

I.1.1 Pathology of biliary tract cancers

With the exception of small duct type iCCA, biliary tract cancers roughly share the same histological features (2).

Most biliary tract cancers are adenocarcinomas, i.e. malignant epithelial tumors presenting a glandular differentiation. Large duct-type iCCA, pCCA, dCCA and GBC usually present with a tubular pattern whose glands are variably sized and located in an abundant fibrous stroma. They are composed of small to medium sized carcinoma cells with a columnar to cuboidal morphology and a pale or eosinophilic cytoplasm. Mucus secretion, perineural and lymphatic invasions with subsequent lymph node metastases are frequent in these cancers (3-5) (Figure 2). It should be noted that these histological features are also shared by pancreatic ductal adenocarcinoma making the differential diagnosis between an iCCA (large duct type) and a liver metastasis derived from a pancreatic ductal adenocarcinoma, a GBC or an eCCA challenging.

In 2019, large duct- and small duct types iCCA were recognized as distinct in the WHO classification of digestive tumors (2). Unlike large duct type iCCAs which are mainly located near the hilum (proximal to the right and left hepatic ducts), small duct type iCCAs preferentially develop at the liver periphery. The small duct type presents with a mass-forming pattern as opposed to the large duct type iCCA which initially spreads along the large portal tracts according to a periductal infiltrating pattern. This infiltration leads to bile duct wall thickening associated with perineural and/or lymphatic invasion. Eventually, large-duct type iCCA can give rise to a mass-forming tumor (6) (Figure 2).

In small duct type iCCAs, two different components can be variably admixed: the small ductal and the ductular components. The small ductal component refers to tubular glands with cuboidal to low columnar cells having scant cytoplasm; the ductular component is made of cord-like structures showing slit-like lumens and resembles a regenerative ductular reaction (Figure 2). A small

duct type iCCA is called cholangiolocarcinoma when more than 80% of the tumor presents with this ductular morphology. In addition, small duct type iCCA glands are non-mucin secreting and perineural invasions are less frequent than in large duct type iCCA (3).

In 2012, Komuta et al. identified the expression of Mucin1 (MUC1; synonym: epithelial membrane antigen (EMA)) at the apical membrane in the ductular areas of small duct type iCCA. This is in contrast with the intense cytoplasmic MUC1 positivity of large duct type iCCA, called mucin-producing CCA in this study (7). These two different morphological subtypes of iCCA are associated with distinct prognoses, the small duct type presenting a higher 5-year post-surgical survival rate than large duct type (3).

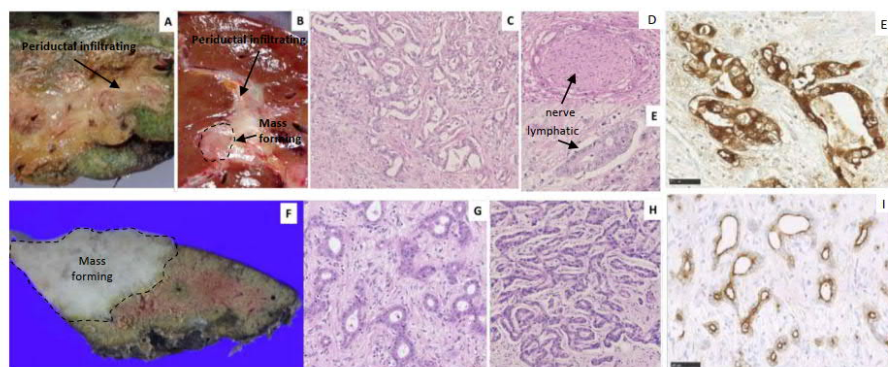


Figure 2: Morphological features of large duct type (A-E') and small duct type iCCAs (F-I). (A-E') Large duct type iCCA shows a macroscopic periductal infiltrating growth pattern leading to a thickening of the bile duct wall in the portal tract (A and B). This periductal infiltrating growth pattern can be admixed with mass forming pattern in large duct type iCCA (B). Large duct type iCCA is composed of variably sized glandular structures in a dense fibrous stroma (C). Perineural and lymphatic invasions are common (D and E). MUC1 expression is cytoplasmic in large duct type iCCA (E'). (F-I). Small duct type iCCA presents with a macroscopic mass forming pattern (F). Tumor glands present distinct configurations: either small tubular glands with scant cytoplasm (G) and/or cord-like structures with slit-like lumen (H). In small duct type iCCA, EMA stains the apical pole. Adapted from Komuta M. Clin Mol Hepatol 2022; 28:396-407.

I.1.2 Pathology of biliary tract cancer precursor lesions

Epithelial dysplasia refers to non-invasive intraepithelial lesions characterized by variable degrees of cytological atypia and architectural abnormalities and presenting a malignant potential. In other words, a dysplastic epithelium does not have all the characteristics of a carcinoma but can be considered as a transforming epithelium potentially able to degenerate. To address the mechanisms of tumor initiation, we decided to focus on the dysplastic lesions, i.e. precursor/premalignant lesions described in the biliary tract.

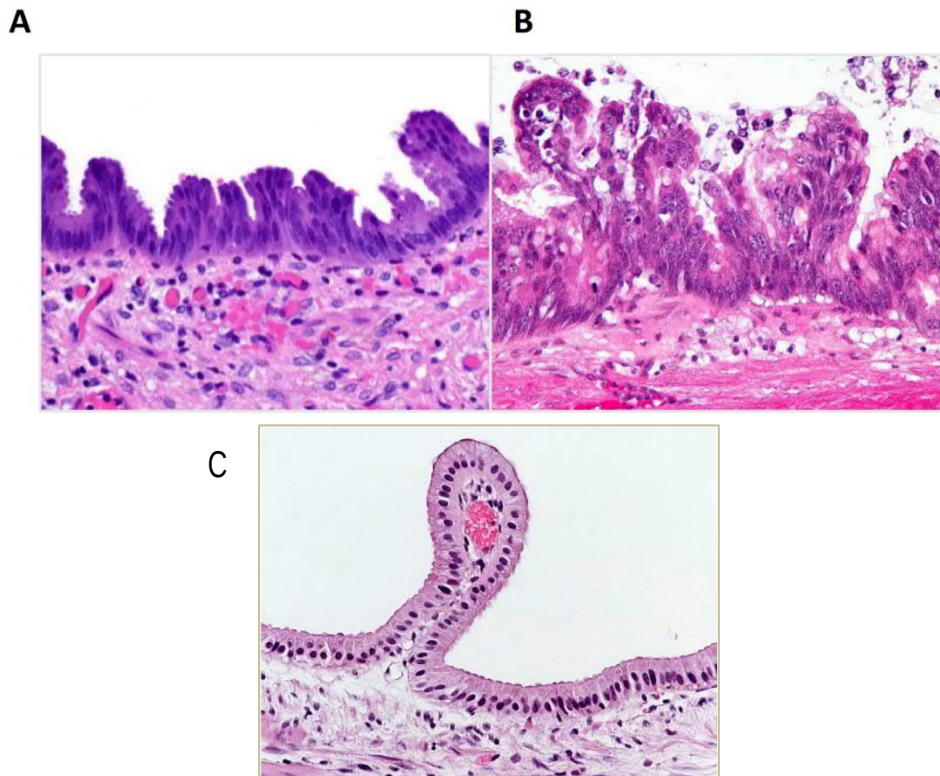
The main precursor lesions of biliary tract cancers are biliary intraepithelial neoplasia (BillIN) and intraductal papillary neoplasm of the bile ducts (IPNB) or intracholecystic papillary neoplasm (ICPN) of the gallbladder. BillIN are considered premalignant lesions of large-duct type iCCA, pCCA and dCCA as well as GBC. Precursor lesions of small-duct type iCCA remain currently unknown. IPNBs affect the bile ducts and are recognized as precursor lesions of iCCA, pCCA and dCCA. ICPN is the counterpart of IPNB in the gallbladder. Given their histological similarities, these lesions are described together.

BillINs consist of microscopic, flat or micropapillary lesions, confined to the lumen of the bile ducts or of the gallbladder. Given their microscopic nature, they are usually incidentally diagnosed on resection specimens performed for other reasons. BillINs are categorized into low grade or high grade BillIN based on the highest degree of cytological and architectural atypia (Figure 3). This two-tiered classification replaces the previous three-tiered system defining BillIN-1, BillIN-2 and BillIN-3. BillIN-1 and BillIN-2 are now considered as low grade BillIN and BillIN-3 is classified as high grade BillIN. High grade BillIN and carcinoma in situ are synonymous but the terminology high grade BillIN is preferred. Low grade BillIN is morphologically characterized by mild cytoarchitectural atypia with pseudostratification of the nuclei, increased nuclear-cytoplasmic ratio and hyperchromasia. High grade BillIN is defined by a loss of nuclear polarity and marked cytological atypia. High grade BillIN usually shows more complex patterns such as micropapillae (Figure 3) (8).

Low and high grade BillIN can present with different cell phenotypes including biliary, intestinal and gastric features but these cellular aspects do not have any known prognostic impact. In the gallbladder, BillIN can occur in Rokitansky-

Aschoff sinuses, i.e. epithelial diverticulæ into the muscular layer, which should not be confused with invasive carcinoma (8).

Figure 3: Histological features of BillIN. (A) BillIN low grade, (B) BillIN high grade. Courtesy of O. Basturk, WHO Classification of Tumours Editorial Board. Digestive system tumours. Lyon (France): International Agency for Research on Cancer; 2019. WHO classification of tumours series, 5th ed.; vol. 1. <https://publications.iarc.fr/601>. These lesions are compared to a normal gallbladder epithelium(C). From Hansel, Current Diagnostic Pathology, 2004, page 305.



IPNBs are grossly visible intraductal polypoid lesions with a predominant papillary architecture (Figure 4). As mentioned before, they affect intra- and/or extrahepatic bile ducts and present sometimes as multifocal lesions (9). These intraductal masses are usually associated with an upstream dilation of the bile ducts due to ductal obstruction by the lesion itself and/or by an excessive mucine secretion. If IPNB secretes a lot of extracellular mucins, the dilatation affects bile ducts located proximally and distally to the lesion. Extremely dilated bile ducts can become cystic and such IPNB should not be confused with mucinous cystic neoplasms (MCN) (cfr infra). Given their mass-forming nature potentially creating bile duct obstruction, patients affected with IPNB can suffer from abdominal pain, cholestasis and cholangitis whereas BillIN remains asymptomatic. The presence of an intraductal mass associated with bile duct dilations can also be detected by medical imaging. Histologically, IPNBs are characterized by a predominant papillary pattern. The papillae consist in epithelial projections sustained by fibrovascular cores (Figure 4) (9).

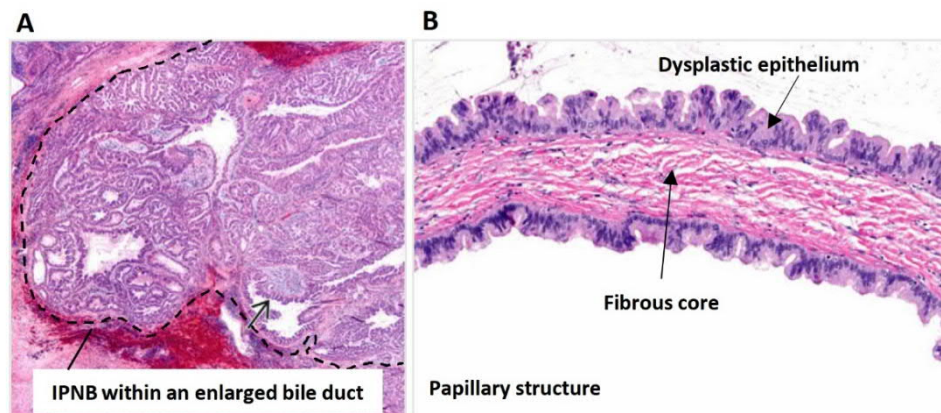


Figure 4. Histological features of IPNB. (A) IPNB filling and dilating an intrahepatic bile duct. (B) Papillary structure from an IPNB supported by a fibrous core and covered by a dysplastic epithelium (low grade dysplasia in that case. Adapted from Westerhoff, M., Lamps, L., Kakar, S., Diagnostic pathology hepatobiliary and pancreas, third edition, elsevier, p318-319.

This papillary pattern can be mixed with tubular components which can become more abundant and are occasionally called “intraductal tubular or tubulopapillary neoplasms” instead of IPNB by comparing with the well established pancreatic intraductal tubulopapillary neoplasm (ITPN). However, pancreatic ITPN and intraductal papillary mucinous neoplasm (IPMN) are considered distinct entities because they present distinct genomic features. Such data are not available yet in the bile duct lesions, thereby preventing us to consider “intraductal tubular/tubulopapillary neoplasms” as distinct from IPNB. IPNB can present with low grade or high grade dysplasia and the criteria used for this classification are the same as for BillIN. The epithelium lining IPNB can present with distinct cellular phenotypes, namely intestinal, biliary, gastric and oncocytic phenotypes. The gastric type epithelial cells express the mucins MUC5AC and MUC6, whereas the intestinal and pancreatobiliary IPNB are MUC2- and MUC1-positive, respectively. Gastric and intestinal IPNB are most common in Asia, while the pancreatobiliary subtype predominantly affects the western countries. The latter seems to be associated with pejorative histological features and worse clinical outcomes. Of note, the Japan-Korea Cooperative study group subclassified IPNB based on their similarities to their pancreatic counterpart (IPMN). Type 1 IPNB usually concerns the intrahepatic bile ducts and are histologically similar to IPMN with mucin hypersecretion. Type 2 IPNB is located extrahepatically; it is not associated with mucin hypersecretion and presents a worse postoperative outcome (9).

ICPNs are the gallbladder's counterpart of IPNBs and refer to macroscopically visible mass-forming tumors protruding into the lumen of the gallbladder (Figure 5) (10). Approximately 50% of the patients affected by ICPN suffer from right upper quadrant pain. Microscopically, the predominant architectural pattern of ICPN is a papillary configuration possibly associated with tubular units (Figure 5). As for IPNB, several cytological phenotypes (biliary, gastric, intestinal and oncocytic) have been described but the cellular phenotypes displayed by ICPN are more heterogeneous as compared to IPNB and the different cell types are frequently mixed, making this subclassification challenging. Moreover, these various morphological subtypes do not have any known clinical impact. The more important is to define the degree of dysplasia based on the highest degree of cytoarchitectural atypia within the lesion. Again, low grade dysplasia is characterized by mild to moderate cytological atypia while high grade dysplasia displays loss of nuclear polarity, more severe nuclear polymorphism and higher

architectural complexity. Globally, ICPN are less frequent than BilIN in the gallbladder and only 6% of GBC develop from ICPN (10). The main carcinogenic pathway in the gallbladder thus remains the BilIN-carcinoma sequence.

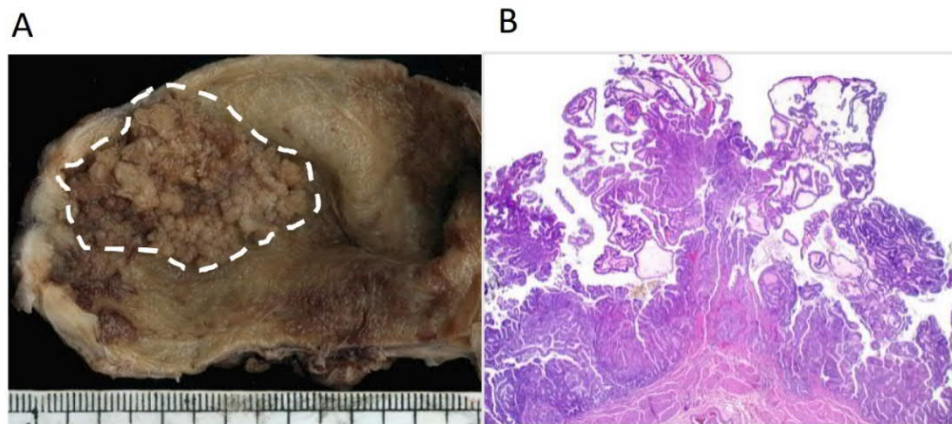


Figure 5: Macroscopic (A) and microscopic (B) appearance of ICPN. Courtesy of O. Basturk, WHO Classification of Tumours Editorial Board. Digestive system tumours. Lyon (France): International Agency for Research on Cancer; 2019. WHO classification of tumours series, 5th ed.; vol. 1. <https://publications.iarc.fr/601>.

Mucinous cystic neoplasms (MCN) are also considered premalignant lesions of the biliary tract but they are less frequent than BilIN and IPNB. They occur in women and mainly concern the liver even if some cases of MCN have been reported extrahepatically and exceptionally in the gallbladder. Histologically, they consist of cystic lesions without any communication with the bile ducts, lined by a cuboidal to columnar and variably mucin-producing epithelium supported by an ovarian-like stroma (Figure 6). The absence of communication with the bile ducts and the presence of an ovarian-like stroma help to differentiate them from IPNBs. Most of these MCN present low grade dysplasia but rare cases have high grade dysplasia or associate with an invasive adenocarcinoma (11).

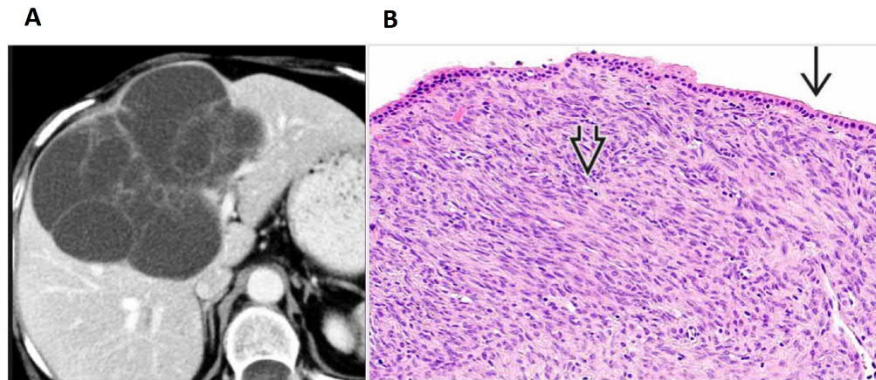


Figure 6: Mucinous cystic neoplasm. (A) Axial abdominal CT scan showing a multilobulated cyst in the liver. (B) Cyst wall lined by a columnar epithelium (here without dysplasia) (thin arrow) and sustained by a dense ovarian-like stroma (thick arrow). Adapted from Westerhoff, M., Lamps, L., Kakar, S., Diagnostic pathology hepatobiliary and pancreas, mucinous cystic neoplasm, liver, third edition, elsevier, p308.

Finally, the role of von Meyenburg complexes in the carcinogenesis of iCCA is a matter of debate. These complexes are also called biliary microhamartoma and consist of multiple and small nodules related to portal tracts and composed of irregular ductal structures embedded in a fibrous stroma (Figure 7) (12). They are usually considered benign lesions resulting from ductal plate malformations but

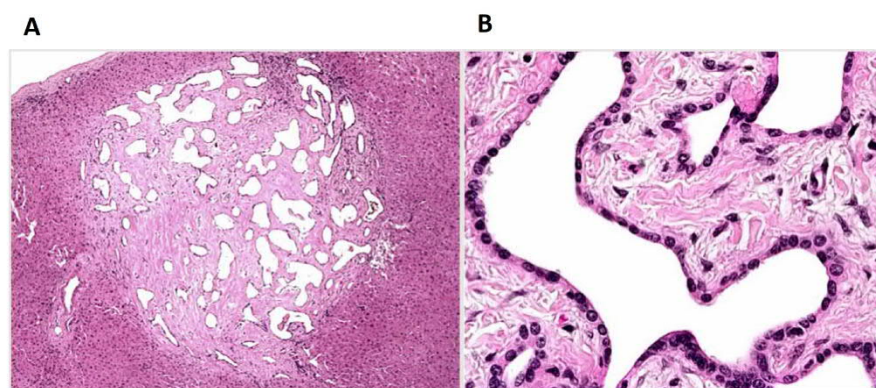


Figure 7: von Meyenburg complex. (A) Low power magnification: small liver nodule composed of branching ductal structures embedded in a fibrous stroma. (B) High power magnification: ductal structures lined by a flattened epithelium without cytological atypia. Adapted from Westerhoff, M., Lamps, L., Kakar, S., Diagnostic pathology hepatobiliary and pancreas, von Meyenburg complex (biliary microhamartoma), third edition, elsevier, p304.

rare histological evidences suggest that von Meyenburg complexes may evolve into ICCA (13).

Since BillIN and IPNB/ICPN are the most frequent and the best established precursor lesions in the biliary tract, we decided to focus our analyses on these lesions while MCN and von Meyenburg complexes are beyond the scope of the thesis.

I.2 Pathogenesis of biliary tract cancers

In 2015, Nakamura et al. uncovered different molecular spectra in biliary tract cancers depending on their location along the biliary tree (14). This revealed the molecular heterogeneity of the different subtypes of tumors and justified to consider iCCA, eCCA and GBC as different entities (1). In their large scale study, 260 tumors including 145 iCCA, 86 eCCA and 29 GBC from Japanese patients were submitted to whole exome and transcriptome sequencings. This identified several mutational signatures and distinct repertoires of altered cancer driver genes (Figure 8).

With the exception of TP53, BRCA1-BRCA2 and PI3KCA mutations commonly found in all biliary tract cancer subtypes, the repertoire of altered cancer driver genes varied between different tumor subtypes. KRAS, SMAD4, ARID1A and GNAS mutations were detected both in iCCA and eCCA, but not in GBC. At the opposite, GBC harbored mutations in EGFR, ERBB3, PTEN, ARID2, MLL2, MLL3 and TERT promoter which were not found in the CCA cohort. Among CCA, FGFR2 fusions, IDH1/2, EPHA2 and BAP1 mutations were specifically found in iCCA whereas PRKACA and PRKACB fusions, ELF3 and ARID1B mutations occurred extrahepatically. (14) (Figure 8)

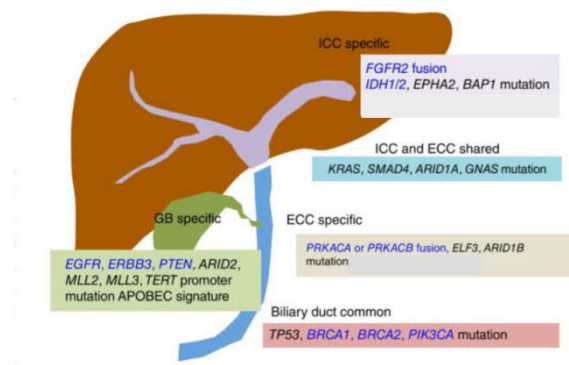


Figure 8: Mutational spectrum in biliary tract cancers. ICC= intrahepatic cholangiocarcinoma, ECC= extrahepatic cholangiocarcinoma, GB= gallbladder. Adapted from Nakamura et al. Nat Genet 2015;47:1003-1010.

Nakamura and coworkers analyzed the occurrence of a mutational signature associated with the activity of a group of enzymes able to induce DNA mutations, called the APOBEC-mediated mutational signature, in the different subtypes of BTC. They found a higher incidence of APOBEC-mediated mutational signature in GBC as compared to CCA and more frequently in eCCA than in iCCA (Figure 8). The occurrence of this APOBEC-mediated mutational signature correlated with the expression of APOBEC3B, a member of this enzyme family which displays cytidine deaminase activity and constitutive nuclear localization. Several arguments allow to consider that overexpression of APOBEC3B can induce genomic DNA damage and mutations leading to cancer (14).

Given these distinct mutational spectra, we decided to describe separately risk factors and molecular alterations occurring in CCA and GBC.

I.2.1 Pathogenesis of cholangiocarcinomas

I.2.1.1 Epidemiology and risk factors of cholangiocarcinomas

CCA is considered a rare cancer in most countries with an incidence of less than 6/100,000 inhabitants. This incidence is higher in East Asia (China, South Korea) and exceptionally high in northeast, north and central Thailand, where it reaches 85/100,000 inhabitants (Figure 9) (1, 15).

East Asia, northeast, north and central Thailand are characterized by a high rate of liver fluke infections (*Opisthorchis viverrini* and *Clonorchis sinensis*) which are recognized as definite cause of CCA in these regions (16, 17). Liver flukes are flatworm parasites transmitted by freshwater fish. People get infected by eating undercooked freshwater fish containing the encysted larval parasite which is digested by gastric and intestinal juices. Following this digestion, the juvenile flukes migrate through the ampulla of Vater into the common bile duct and intrahepatic bile ducts where they mature, reproduce and can live for many years (17).

In Western countries, primary sclerosing cholangitis (PSC) is the best known risk factor of CCA (15). PSC is a cholestatic liver disease characterized by intra- and/or extrahepatic strictures resulting from bile duct inflammation and fibrosis. Although the etiology of the disease has not been elucidated yet, several genetic and environmental features might be involved in its pathogenesis. Given that 10 to 20% of PSC patients develop CCA, PSC is considered a premalignant disease, in which the accumulation of bile acids might contribute to accelerate tumor initiation and/ or progression (18).

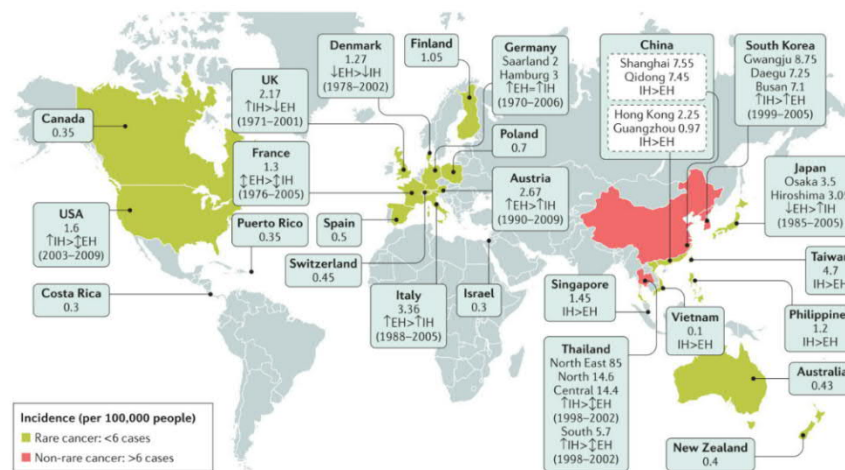


Figure 9 : Worldwide incidence of CCA. Green identifies countries with incidence lower than 6 per 100 000 inhabitants (rare cancer), whereas pink labels countries in which CCA is more common (>6 per 100 000 inhabitants). When available, the relative incidence of intrahepatic (IH) versus extrahepatic (EH) CCA, as well as the trend of incidence are indicated. From Banalles et al. Nat Rev Gastroenterol Hepatol 2016;13:261-280.

Liver fluke infection and PSC account for only a minority of CCA cases, and most CCA occur de novo without known predisposing condition. In their recent meta-analysis, Clements et al. identified additional risk factors for both iCCA and eCCA (15). Cirrhosis, viral hepatitis (hepatitis B and C), inflammatory bowel disease and type 2 diabetes increase the risk of CCA with a greater association with iCCA than eCCA. Choledocal cyst, lithiasis and smoking are more often associated with eCCA than with iCCA. Alcohol consumption may favor both eCCA and iCCA.

Additional risk factors have been described and are summarized in the table below.

Risk factor	Study type	OR or RR from selected studies
Choledochal cyst ³⁰	Meta-analysis	OR 26.71 for iCCA OR 34.94 for eCCA
Choledocholithiasis ³⁰	Meta-analysis	OR 10.08 for iCCA OR 18.58 for eCCA
Cholelithiasis ³⁰	Meta-analysis	OR 3.38 for iCCA OR 5.92 for eCCA
Cholecystolithiasis ³⁰	Meta-analysis	OR 1.75 for iCCA OR 2.94 for eCCA
Caroli disease ³⁹⁶	Population-based study	OR 38 for iCCA OR 97 for eCCA
Primary sclerosing cholangitis ³⁹⁶	Population-based study	OR 22 for iCCA OR 41 for eCCA
Cirrhosis ³⁰	Meta-analysis	OR 15.32 for iCCA OR 3.82 for eCCA
Chronic hepatitis B ³⁰	Meta-analysis	OR 4.57 for iCCA OR 2.11 for eCCA
Chronic hepatitis C ³⁰	Meta-analysis	OR 4.28 for iCCA OR 1.98 for eCCA
Haemochromatosis ³⁹⁶	Population-based study	OR 2.1 for iCCA
Inflammatory bowel disease ³⁰	Meta-analysis	OR 2.68 for iCCA OR 2.37 for eCCA
Chronic pancreatitis ³⁹⁶	Population-based study	OR 2.7 for iCCA OR 6.6 for eCCA
Liver fluke (<i>Opisthorchis viverrini</i> , <i>Clonorchis sinensis</i>) ³⁹⁷	Meta-analysis	OR 5 iCCA > eCCA
Type 2 diabetes mellitus ³⁹⁸	Meta-analysis	OR 1.73 for iCCA OR 1.5 for eCCA
Nonalcoholic fatty liver disease ³⁹⁹	Meta-analysis	OR 2.2 for iCCA OR 1.5 for eCCA
Obesity ³⁰	Meta-analysis	OR 1.14 for iCCA OR 1.2 for eCCA
Hypertension ³⁰	Meta-analysis	OR 1.10 for iCCA OR 1.21 for eCCA
Alcohol consumption ³⁰	Meta-analysis	OR 3.15 for iCCA OR 1.75 for eCCA
Cigarette smoking ³⁰	Meta-analysis	OR 1.25 for iCCA OR 1.69 for eCCA
Environmental toxins		
Thorotrast (banned 1969) ^{400,401}	Retrospective study	RR >300
1,2-Dichloropropane ⁴⁰²	Retrospective study	RR 15
Asbestos ⁴⁰³	Case-control study	OR 4.8 for iCCA OR 2.1 for eCCA
Asbestos ⁴⁰⁴	Case-control study	OR 1.1–1.7 for iCCA No association with eCCA

eCCA, extrahepatic cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma; OR, odds ratio; RR, relative risk.

Table 1: Risk factors for CCA. From Banales, Nature reviews gastroenterology and Hepatology, 2020.

CCA can thus develop in a context of liver diseases that mainly affect hepatocytes (i.e. hepatitis B and C and non biliary cirrhosis). I therefore speculate that the small-duct type iCCAs originate from hepatocytes undergoing transdifferentiation as described in mice (19).

I.2.1.2 Genomic alterations in cholangiocarcinomas

Beside TP53 and KRAS mutations that appear to be the most frequent in iCCA and eCCA (14, 20), a wide panel of genes has been reported to be mutated in CCA. There is important interpatient variability and the studies usually do not mention the risk factors to which CCA patients were exposed nor the histological subtype of iCCA. Moreover, the gene panel included in the targeted sequencings is not standardized. For these reasons, it is impossible to define specific mutational spectra associated with distinct risk factors or with specific histological subtypes of iCCA (large duct type versus small duct type iCCA).

In that context, I decided to focus on the most frequent and/or the druggable mutations and to describe the functional evidences supporting their oncogenic effect (Table 2).

CCA	Frequent mutations	Functional evidences for a oncogenic/ tumor suppressive effect in CCA biology
iCCA	p53 mutations (higher incidence in liver-fluke associated and HBV associated iCCA)	<i>Alb-Cre/loxP-Stop-loxP Kras^{G12D}/Tp53^{loxP/loxP} induces iCCA in mice</i>
	KRAS mutations	<i>Alb-Cre/loxP-Stop-loxP Kras^{G12D}/Tp53^{loxP/loxP} induces iCCA in mice</i> <i>Alb-Cre/loxP-stop-loxP-Kras^{G12D}/Pten^{loxP/loxP} induces iCCA in mice</i> <i>K19-Cre^{ERT2}/loxP-stop-loxP-Kras^{G12D}/Pten^{loxP/loxP} induces iCCA in mice</i>
	FGFR2 fusions (enriched in small duct type iCCA)	FGFR2 fusions promote anchorage-independent growth <i>in vitro</i> and tumor growth in xenografts mouse models
	IDH1-IDH2 mutations (enriched in small duct type iCCA)	NA
	SMAD4 deletions/non sense mutations	NA
	PI3KCA mutations	NA
eCCA	p53	NA
	KRAS	<i>K19-Cre^{ERT2}loxP-Stop-loxP Kras^{G12D} / Tgfb^{R2}^{loxP/loxP} E-Cadherin^{loxP/loxP} induces eCCA in mice</i>
	SMAD4 deletions/non sense mutations	NA
	PI3KCA mutations	NA

Table 2: Frequent genomic alterations in CCA. NA= non available. The druggable mutations are in red.

As mentioned, TP53 and KRAS mutations are the most frequent in both iCCA and eCCA. KRAS is mutated in 16 to 25% of iCCA and in 12% of eCCA whereas TP53 mutations are found in 23 to 38% of iCCA and 26% of eCCA (14, 20).

TP53 mutations correlate with worse survival in iCCA (20) and usually consist in non-identical missense mutations targeting the p53 DNA-binding domain (14, 20). The TP53 mutational landscape in eCCA is less well characterized and seems rather variable (14). The rare studies considering the etiology of the disease found a higher incidence of TP53 mutations in hepatitis B virus associated iCCA and in liver-flukes associated CCA as compared to CCA unassociated with these infections (20). Unfortunately, because most of the genomic studies are not standardized, it is not possible to associate a specific etiology with a specific mutational signature.

Different mouse models have shown the involvement of activating Kras^{G12D} mutations in iCCA and eCCA tumorigenesis, in combination with mutations in other genes. The induction of Kras^{G12D} associated with Tp53 or Pten deletion in Alb-Cre/loxP-Stop-loxP Kras^{G12D}/Tp53^{loxP/loxP}, Alb-Cre/loxP-stop-loxP-Kras^{G12D}/Pten^{loxP/loxP} or K19-Cre^{ERT2}/loxP-stop-loxP-Kras^{G12D}/Pten^{loxP/loxP} mouse models gives rise to iCCA and other liver tumors depending on the cell type(s) targeted by this induction and the timing of the induction (21, 22). Similarly, the induction of a Kras^{G12D} mutation associated with Tgf β 2 and E-Cadherin deletions using K19-Cre^{ERT2} induces eCCA along the common bile duct (23). Despite the development of these mouse models, KRAS^{G12D} biology in the specific context of CCA development has never been studied and its effects are deduced from the common knowledge of KRAS^{G12D} mutations on the MAPK pathway (24).

FGFR2 fusions proteins generated by chromosomal translocations were almost exclusively reported in iCCA with a frequency of 10-15% and are enriched in the small duct type iCCA (6). They consist of fusions with variable partners, leading to forced dimerization and constitutive tyrosine-kinase activity of the dimerized receptor, and activation of the downstream MAPK pathway (25, 26). FGFR2 fusions and KRAS mutations are mutually exclusive which leads to consider FGFR2 fusions as cancer drivers in iCCA (26). They promote anchorage-independent growth in vitro and tumor growth in xenografts mouse models (25). Today, FGFR-tyrosine

kinase inhibitors induce clinical responses in iCCA patients harboring the fusion and Pemigatinib is used in previously treated locally advanced or metastatic CCA (27) .

IDH1 and IDH2 mutations concern exclusively iCCA. IDH1 mutations are reported in 13% of iCCA patients (28). Although they are unusual in other carcinomas, these gain-of-function mutations are known in acute myeloid leukemia, glioblastoma and chondrosarcoma. Wild type IDHs (isocitrate dehydrogenases) are metabolic enzyme that convert isocitrate in α -ketoglutarate and are involved in several metabolic pathways determinant for glucose sensing, glutamine metabolism, lipogenesis and regulation of redox states (29). The reported IDH1-2 missense mutations in CCA lead to a new enzymatic activity producing high levels of the oncometabolite, 2-hydroxyglutarate. 2-hydroxyglutarate is not involved in the normal metabolic pathways regulated by IDHs but instead, inhibits other enzyme that use α -ketoglutarate as co-enzyme, i.e. mainly histone and DNA demethylases. (Figure 10)

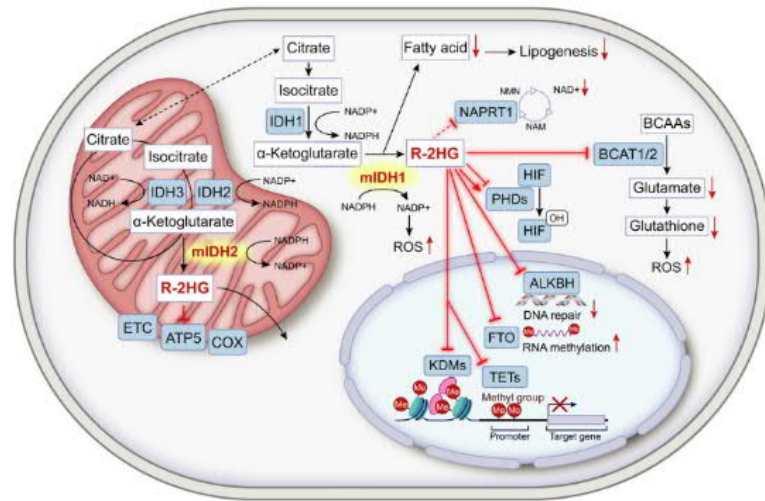


Figure 10: Function of wild type and mutant IDH. "The metabolic reactions catalyzed by the (...) IDH isozymes are shown. The WT IDH enzymes interconvert α KG and isocitrate. The cancer-associated IDH1 and IDH2 cause altered enzymatic activity, using NADPH to reduce α KG to (R)-2HG. (R)-2HG acts as a competitive inhibitor of α KG-dependent enzymes, including KDMs and TET family DNA demethylating enzymes as well as many other dioxygenases implicated in RNA demethylation, collagen maturation, and other functions". From Wu, Hepatology, 2022.

Thus, IDH1 and IDH2 gain-of-function mutations induce complex epigenetic modifications and IDH mutant CCA present distinct gene expression and methylation profiles as compared to non-mutant IDH CCA. The panel of genes targeted by these modifications is not fully established, but ARID1A which is involved in chromatin remodeling, seems to be a recurrent target. Indeed, promoter silencing methylations occur in IDH-mutant CCA (30). Beside these epigenetic modifications, complex metabolic dysfunctions that are not fully understood occur in IDH-mutant cells (29). They include an increased production of reactive oxygen species and altered regulation of the oxidative phosphorylation in the mitochondria (29, 30). Anyway, IDH1 inhibitors such as Ivosidenib, targeting the mutated IDH1, give promising responses in clinical trial (28)

BRAF mutations are reported in only 1-3% of the cases and mostly concern iCCA. Even if they are unusual, these mutations are important to consider because most BRAF mutations consist in oncogenic BRAF^{V600} mutations that can be targeted by currently available inhibitors (Vemurafenib and Dabrafenib). The oncogenic effect of such mutations is mediated by the activation of the MEK/ERK module (31).

1.2.1.3 Signaling pathways active in cholangiocarcinoma

Distinct lines of evidence support the activation of several signaling pathways during cholangiocarcinogenesis (Table 3).

Signaling pathway	CCA	Evidence(s) supporting an oncogenic/ tumor suppressive effect in CCA
IL6/JAK/STAT3	iCCA CCA CCA	STAT3 and p-STAT3 overexpression correlate with worse prognosis STAT3 overexpression in CCA cell lines promotes proliferation, survival and tumor growth SOCS3 hypermethylation is found in human CCA
TGFβ/SMAD4 Complex effects	CCA eCCA	TGFβ1 induces EMT in CCA cell lines TGFβR2 deletion induces eCCA in mice
ERBB signaling	iCCA + eCCA CCA	Overexpression of ERBB1 to ERBB4 correlates with worse prognosis EGFR/ERBB1 and ERBB2 activations promote proliferation, migration and invasion
Histamine	CCA	H1R and H2R activation increases proliferation of CCA cells
PI3K/AKT (PTEN= inhibitor)	iCCA iCCA iCCA	Alb-Cre/loxP-stop-loxP-Kras ^{G12D} /Pten ^{loxP/loxP} induces iCCA in mice K19-Cre ^{ERT2} /loxP-stop-loxP-Kras ^{G12D} /Pten ^{loxP/loxP} induces iCCA in mice Transposase-mediated expression of AKT (+YAP and IL-33) in cholangiocytes induces iCCA in mice
Notch (Notch intracellular domain) (HES-1= target gene)	iCCA iCCA eCCA	Notch1 increases proliferation and survival of iCCA cells Induction of Notch intracellular domain in hepatocytes generates iCCA in mice HES-1 is overexpressed in human eCCA
Hedgehog (cyclopamine=inhibitor of SMO)	CCA	Cyclopamine reduces CCA proliferation, migration, invasion and tumor growth
Wnt/B-Catenin	CCA	Tumor-associated macrophages produce Wnt7B ligand

Table 3. Signaling pathway active in CCA

Interleukin-6 (IL-6) shows a tumor-promoting role in CCA, in line with the recognition of inflammatory conditions as risk factors for CCA. In iCCA, IL-6 is produced by activated Kupffer cells, tumor-associated macrophages, cancer-associated fibroblasts (CAF) and the CCA epithelial cells (24). IL-6 binds to its membrane receptor and signals via the activation of the JAK/STAT3, MAPK and PI3K/AKT pathways (32). This results in proliferation (32) and cell death inhibition via the activation of MCL1, an antiapoptotic gene from the BCL2 family (33). Several evidences allow to consider an oncogenic effect for IL-6: (i) STAT3 and p-STAT3 expression is increased in iCCA and correlates with worse prognosis (34, 35), (ii) STAT3 overexpression in CCA cell lines increases proliferation, cell survival and tumor growth in xenograft mouse models (35), (iii) SOCS3 promoter hypermethylations are found in CCA, leading to SOCS3 silencing and subsequent activation of the JAK/STAT3 pathway (36)(Figure 11).

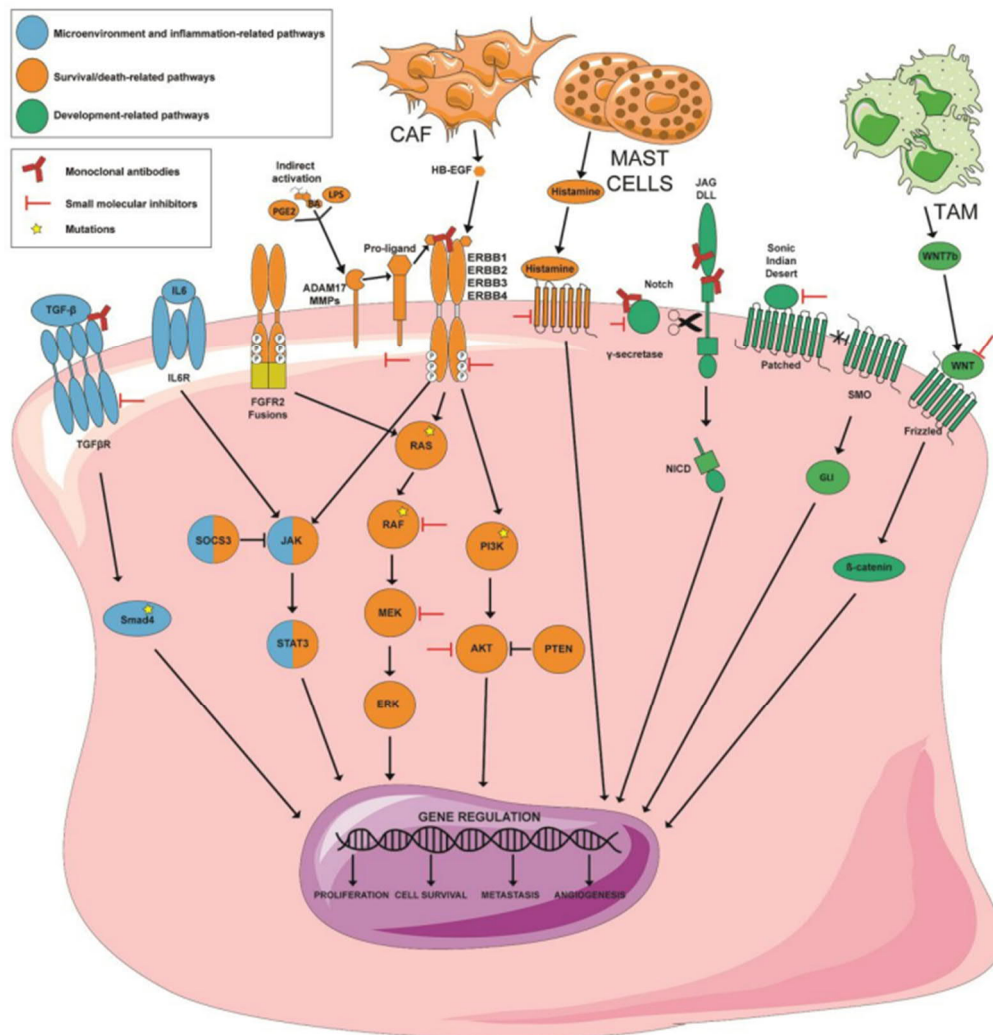


Figure 11: Molecular alterations in CCA . From Fouassier et al. Liver Int 2019;39 Suppl 1:43-62.

The TGF β /SMAD4 pathway is involved in cholangiocarcinogenesis in a complex manner with both oncogenic and tumor suppressive effects. CCA cell lines treated with TGF β 1 undergo epithelial to mesenchymal transition (EMT) and improve migration and invasion abilities (37, 38). At the opposite, SMAD4 mutations including deletions and non-sense mutations are reported in CCA (14) and SMAD4 expression is lost in some iCCA at the protein level (39). Tgf β R2

deletion in cholangiocytes contributes to eCCA development in a transgenic mouse model inducing the conditional expression of Kras^{G12D} associated with Tgf β R2 and E-Cadherin deletions in k19 expressing cells (23). Of note, in these experimental conditions, eCCA arises from peribiliary glands and not from the cholangiocytes lining the bile ducts. This is because transgenic cells deleted for E-Cadherin in this model are rapidly eliminated from the epithelium lining the bile ducts whereas they remain longer in the peribiliary glands.

The four receptors of the EGFR family (EGFR/ERBB1, ERBB2, ERBB3, ERBB4) are overexpressed in both iCCA and eCCA which correlates with poor prognostic features (40). Mutations in these receptors are unusual in CCA. While the oncogenic mechanisms of ERBB3 and ERBB4 activations in CCA are poorly known, a huge body of evidence supports the effect of EGFR and ERBB2 activations on CCA proliferation, migration and invasion through the JAK/STAT3, MAPK and PI3K/AKT pathways (40). Interestingly, several compounds involved in cholangiocarcinogenesis indirectly promote EGFR activation. Indeed, bile acids, lipopolysaccharides and prostaglandin E2 bind to their membranous receptor (TGR5, TLR4 and EP1, respectively) promote intracellular signaling pathways leading to the activation of metalloproteinases and the latter are able to cleave some EGFR proligands expressed on the epithelial cell to release mature EGF and subsequently activate the EGFR pathway. Another source of ligands for the EGFR are CAF releasing HB-EGF that activates EGFR on the CCA cell (Figure 11). In turn, CCA tumor cells produce TGF β which increases HB-EGF secretion by CAF (41).

There are reasons to consider an impact of histamine on CCA progression. CCA cells express the histamine receptors (H1 to H4 histamine receptors), produce histamine and attract mast cells, a major source of histamine, in their microenvironment (42) (Figure 11). The activation of H1 and H2 histamine receptors shows a stimulatory effect on cholangiocyte proliferation both in cholestatic and CCA conditions (43). However, it remains unclear if targeting the histamine signaling pathway could constitute a therapeutic option in CCA (24). Data from mouse models of PSC suggest a role of histamine receptor antagonists in iCCA chemoprevention in high risk patients (42).

The oncogenic effect of the PI3K/AKT pathway in liver results in enhanced cell survival. PI3K gain-of-function mutations are widely described in CCA and some

mouse models confirmed the impact of AKT activation on cholangiocarcinogenesis. Transposase-mediated expression of active AKT and YAP in cholangiocytes coupled with bile duct ligation and IL-33 administration promotes iCCA development (44). As mentioned above, other mouse models validated the tumor suppressor role of PTEN that antagonizes the PI3K/AKT pathway in cholangiocarcinogenesis (Figure 11).

Several lines of evidence allow to consider the involvement of the Notch pathway in CCA (Figure 11). The Notch receptors as well as their target gene HES-1 are expressed in eCCA and expression of the Notch 1 and 3 ligands correlate with less differentiated tumors (45). Notch 1 increases proliferation and survival of iCCA cells (46). Mouse models inducing the expression of the Notch intracellular domain in hepatocytes using a transgenic Alb-Cre^{ER T2} system or a transposon-mediated method develop iCCA (19, 47). This demonstrates the role of the Notch signaling pathway in CCA tumorigenesis and introduces new insights into the cell of origin of CCA. Indeed, these experimental models show that iCCA can originate from hepatocytes in mice. Determining the cell of origin of CCA is impossible in human because it requires lineage tracing experiments.

Hedgehog signaling is best known for its tissue-patterning role during development but it also contributes to CCA biology likely via an autocrine pathway. CCA cells can indeed produce and respond to SHH (48) (Figure 11). iCCAs overexpress the ligand SHH (49) and the inhibition of the effector SMO using cyclopamine reduces CCA cell proliferation, migration, invasion in vitro and tumor growth in vivo (48). However, these effects could be mediated by a less well characterized non canonical Hedgehog pathway given that the canonical one requires the presence of a primary cilium which is absent from CCA cells (50).

The Wnt/ β -Catenin pathway is active in CCA and tumor-associated macrophages have been identified as a source of Wnt7B ligand inducing the activation of this pathway (51) (Figure 11).

Of note, my thesis focus on epithelial transitions promoting carcinogenesis along the biliary tract. Interactions with the microenvironment (cancer associated fibroblasts, tumor-associated macrophages, immune cells) are definitely determinant in cancer and in BTC characterized by a prominent stromal reaction

(52) (53). However, the role of the microenvironnement falls out of the scope of the present work.

I.2.1.4 Molecular alterations in intra/extrahepatic BilIN and in IPNB

The molecular alterations occurring in precursor lesions are poorly known because most studies analyze full-blown biliary tract cancers. The gap of knowledge is even more important for BilINs as compared to IPNBs because the access to BilIN samples is more difficult given their microscopic and asymptomatic nature.

KRAS mutations at codons 12 and 13 are detected in 33% of intrahepatic BilIN (Table 1). This was uncovered by sequencing of a few target genes from laser capture microdissected BilIN affecting Japanese patients with hepatolithiasis. Interestingly, KRAS mutations were also detected in some peribiliary glands. Of note, GNAS (G-protein alpha-subunit)mutations and TP53 overexpression were also investigated in this study but none of them was identified in intrahepatic BilIN (54) (Table 4).

CCA	BilIN	IPNB	
iCCA large duct type	Kras mutations (Hepatolithiasis, Japan)	<u>Western countries</u> KRAS mutation ⇒ p53 overexpression ⇒ SMAD4 loss of function	<u>Asia</u> GNAS => mucus hypersecretion => better prognosis (« Biliary IPMN ») + KRAS, CTNNB1 mutations
eCCA	No data		

Table 4 : Mutational spectrum of intrahepatic, extrahepatic BilIN and IPNB

The mutational spectrum of extrahepatic BilIN has not been investigated yet and remains unknown.

The mutational landscape of IPNB displays important geographical variations (Table 5). The main difference concerns the incidence of GNAS (G-protein alpha-subunit) mutations reaching 50% in Asia and only 3% in Western countries (55). In Asian cohorts, GNAS mutations detected in IPNB correlate with a macroscopic mucin hypersecretion and an intestinal phenotype. This correlation is

in line with the gain-of-function in this stimulatory G-protein alpha-subunit (GNAS) leading to its constitutive activation followed by an increase in the intracellular levels of cyclic AMP and the subsequent overexpression of mucin genes (55). GNAS mutations and mucin hypersecretion are shared by pancreatic IPMN, which justifies that some authors consider these IPNB as “biliary IPMN”. In some papers, GNAS gain-of-function mutations correlate with KRAS and RNF43 mutations. RNF43 is an ubiquitin ligase inducing the ubiquitination of the Frizzled receptor in the Wnt/ β -Catenin signaling pathway. Loss-of-function mutations in RNF43 activates the Wnt/ β -Catenin pathway and RNF43 is considered as tumor suppressor. Mutations in other actors of the Wnt/ β -Catenin pathway (CTNNB1/ β -Catenin and APC) are reported in asian IPNBs (56, 57) and some β -Catenin mutations are detected in extrahepatic IPNB without GNAS, Kras, RNF43 or APC mutations (57). This led a Taiwanese group to propose a new system of classification for IPNB which complexifies the Japanese-Korean one and introduces a third group for these β -Catenin mutated IPNB (57). In Western countries, GNAS mutations do not seem determinant for the development of IPNB. At the opposite, TP53 overexpression, activating KRAS mutations and p16 inactivation by loss-of heterozygosity or promoter methylation are detected in low grade IPNB and are considered early events in that process, while loss of expression of SMAD4 (assessed by immunohistochemistry) occur at the stage of high grade IPNB and subsequent cholangiocarcinoma. The frequency of nuclear TP53 accumulation increases along the sequence low grade IPNB -high grade IPNB-invasive cholangiocarcinoma (58). These alterations were found in an european cohort including both intra- and extrahepatic IPNB which share the same molecular features except the p16 inactivation exclusively detected in extrahepatic IPNB and combined IPNB (i.e. extrahepatic IPNB combined with intrahepatic IPNB).

I.2.2 Pathogenesis of gallbladder cancers

I.2.2.1 Epidemiology of gallbladder cancer

Rates of GBC are highly variable worldwide with Latin America (Chile) and Asia (North India, Pakistan) experiencing the highest incidence (Figure 12) (59). In Mapuche Indians from South America, mortality rates from GBC exceeds those from breast, cervical and pancreatic cancer and GBC is a public health problem (59).

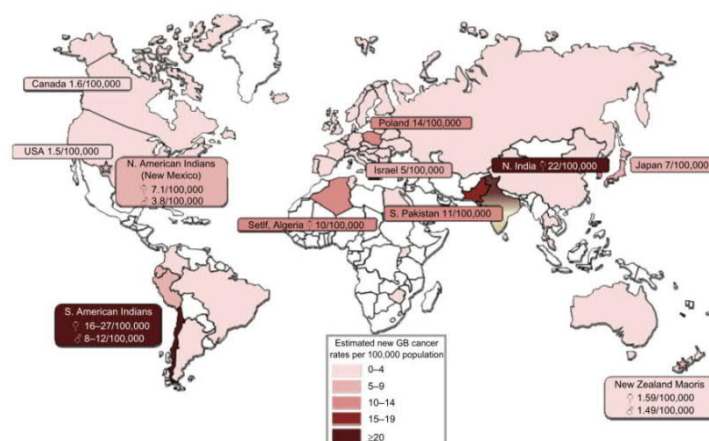


Figure 12: Worldwide incidence of GBC . From Hundal R, Shaffer EA. Gallbladder cancer. Clin Epidemiol 2014;6:99-109.

Epidemiological studies report a positive correlation between the incidence of GBC and the prevalence of gallstone disease. Countries with the highest incidence of GBC display the highest prevalence of gallstone disease (59, 60). This correlation concerns also Mapuche Indians in Chile. 85% of patients suffering from GBC present with a history of chronic gallstone disease. This association is reported for cholesterol gallstones that could favor gallbladder carcinogenesis by creating repeated traumas leading to a local mucosal irritation and/or by inducing chronic

inflammation of the gallbladder wall. Another putative mechanism is the local production of carcinogens derived from bile acids (59).

Cholesterol gallstones develop in bile saturated with cholesterol that cannot be solubilized by bile acids and phospholipids. This imbalance results from excessive secretion of cholesterol and/or decrease in bile salt or phospholipid secretion by the liver. Lithogenic bile, i.e. bile saturated with cholesterol, remains for longer periods in the gallbladder, thereby promoting cholesterol crystallization and the gallstone formation. It impairs gallbladder motility via two different mechanisms: (i) after mucosal absorption, cholesterol disturbs the cholecystokinin signaling cascade and the subsequent gallbladder wall contraction, (ii) lithogenic bile induces chronic inflammation leading to fibrosis of the gallbladder wall, eventually impairing gallbladder contraction and motility (61). In Mapuche Indians who experience high mortality rates associated with GBC, polymorphisms in cholesterol lithogenic genes (for example apolipoproteins and plasma cholesterol ester transfer proteins) that favor cholesterol gallstones are highly prevalent (60).

Although a chronic gallstone disease is the best established risk factor for GBC, less than 3% of patients with symptomatic cholecystolithiasis develop GBC (59), suggesting that unknown additional cofactors are involved in the carcinogenic process.

Several other risk factors for GBC have been described. They are summarized in the table below:

Key risk factors for gallbladder cancer	
Gallstones	Primary sclerosing cholangitis
Age	Family history of gallstones
Sex	<i>Salmonella enterica serovar</i>
Race and ethnicity	Typhi
Gallbladder polyps	Heavy metals
Obesity, diabetes, metabolic syndrome	Diet
Parity	Chemical exposures
Pancreatobiliary maljunction	(for example, to organochlorines)

Table 5. : Risk factors for GBC. From Roa et al., Nature reviews, disease primers, 2022.

Among them, female sex is one of the most significant because GBC affects women 2 to 6 times more often than men. The contribution of female sex hormones to GBC is also suggested by the fact that a higher number of pregnancies increases the risk of GBC (59, 60). Female sex is a known risk factor for cholesterol gallstones because estrogens increase hepatic synthesis and secretion of cholesterol while decreasing bile salt secretion (61). Yet, it remains an independent risk factor for GBC (59, 60).

In Japan, GBC pathogenesis is not associated typically with gallbladder lithiasis but is often linked with pancreatobiliary duct maljunction. The latter is a congenital malformation where the common bile duct and the main pancreatic duct merge before joining the duodenal wall and the sphincter of Oddi. This allows the regurgitation of pancreatic secretions into the common bile duct and gallbladder. No female predominance was noted in this context (59).

Finally, chronic Arsenic exposure due to contamination of the drinking water positively correlates with the incidence of GBC in India (62).

1.2.2.2 Genomic alterations in gallbladder cancer

Little attention was paid to GBC before 2014 when Li and coworkers described frequent mutations in TP53, KRAS and ERBB3. (ref Li) and a significant mutational enrichment in the ERBB signaling pathway.

Several genomic studies followed but the global interpretation of their results suffers from an inter-study variability resulting in part by the limited size of the patient cohorts, the different populations investigated, and the selected sequencing methods. Still, in a rigorous systematic review, Kuipers et al. provide a global overview of the genomic abnormalities found across these studies (63). Implementing well-selected criteria, they analyzed 62 papers that included a total of 3893 GBC samples. Frequent genomic alterations were defined as mutations or copy number variations detected with a frequency of more than 5% in more than 5 samples across all studies. For each genetic alteration, a weighted average of the reported frequencies was calculated based on the sample size of each study. This method allowed to consider 17 frequently mutated and 9 frequently amplified/deleted genes in GBC (Figure 13).

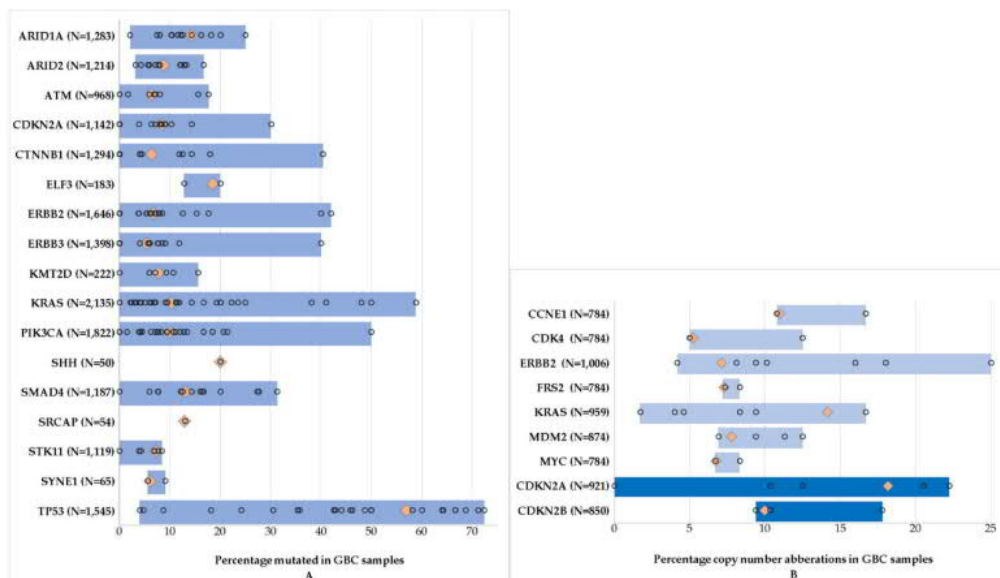


Figure 13: Frequent genetic alterations in GBC. Blue bars represent the minimum and maximum reported alteration frequencies. Dots represent the mutation frequency per study. Orange diamonds represent the weighted average of all frequencies. From Kuipers, Cancers, 2021

Among the most frequent mutation in GBC, those affecting TP53 were still in the lead with a weighted average of 57%, followed by SHH mutations (weighted average: 20%), ELF3 (weighted average: 19%), ARID1A (weighted average: 14%) and SMAD4 (weighted average: 13%). ERBB3, KRAS and PIK3CA mutations are considered frequent in GBC with respective weighted averages of 5.4%, 10.3% and 10%. Surprisingly, KRAS amplifications were also found in some cohorts.

In 2021, after Kuipers' summary paper, Nepal et al. carried out a large scale study on 190 Chinese and Chilean GBC samples. Whole exome and targeted sequencing confirmed the recurrent mutational rate in TP53 (29.3% of the patients) as well as the significant occurrence of mutations in ELF3, ERBB2, PI3KCA and ARID2. Gene set enrichment analysis using the KEGG pathways identified the p53 and Wnt/ β -Catenin pathways as significantly mutated in GBC. However, the enrichment in ERBB signaling found by Li et al. was only present in APOBEC positive cancers. Interestingly, ZNF717, a zinc-finger transcription factor, appears to be the second most frequently mutated gene after TP53; the apoptotic pathway was also significantly mutated (64).

In an attempt to clarify the mutational landscape of GBC, I listed the frequent mutated genes described by the above mentioned studies in table 6. Unfortunately, without standardization of the population studied and of the panel of the genes, it is not possible to associate specific mutations with specific risk factors. The most consistent findings relate to the mutational enrichment in the P53, Wnt/ β -Catenin, apoptosis and ERBB pathways described by Nepal and co-workers.

mutated gene/ pathway	Kuipers, 2021 (various populations)	Nepal, 2021 (chinese and chilean patients)
TP53	+	+
ZNF717	NA	+
SHH	+	-
ELF3	+	+
ARID1A	+	-
ARID2	+	+
SMAD4	+	-
ERBB3	+	-
ERBB2	+	+
PI3KCA	+	+
KRAS	+	-
Wnt/ β -Catenin pathway	NA	+
ERBB signaling pathway	NA	+ (in APOBEC + GBC)
P53 pathway	NA	+
Apoptosis pathway	NA	+

Table 6: Genomic alterations in GBC following Kuipers, cancers, 2021 and Nepal, JHepatol, 2021.

I.2.2.3 Transcriptomic events in gallbladder cancer

Taken together, gallstone-induced GBC presents with a low mutational burden (64) and in this context, transcriptomic analyses could offer another opportunity to uncover key mechanisms driving tumor progression. The RNA-sequencing (bulk sequencing) performed by Nepal et al. on 47 GBC identified 3 transcriptional subtypes based on a 95-gene signature correlating with survival. GBC subtypes 1 and 3 present a poor outcome after resection as compared to subtype 2. Gene set enrichment analyses (GSEA) using the KEGG pathways and the Hallmarks of cancers found a higher expression of the MAPK, the TGF β pathways and of several immune related pathways in subtypes 1 and 3 as well as an enrichment in the EMT, the angiogenic and the hypoxic pathways. This correlates with an increase in the number of CAF, myeloid-derived suppressor cells and endothelial cells in subtypes 1 and 3. The detected T cell exclusion and T cell dysfunction in subtype 3 associated with a higher expression of PD1 and PDL1 suggest that this subtype could exhibit immunosuppression. Finally, subtype 3 shows a hypermethylation context that correlates with hypoxic levels (64). Globally, this new transcriptomic classification highlights the instrumental role of the tumor microenvironment including CAF, T cells and endothelial cells in the progression of GBC. This concept is supported by single-cell RNA sequencing data showing that malignant epithelial cells enriched in mutations in the ERBB signaling pathway and in ERBB3 especially induce an immunosuppressive microenvironment. This is mediated by the secretion of midkine by malignant epithelial cells that favors an immunosuppressive macrophagic differentiation and subsequently recruits T regulatory lymphocytes (65).

I.2.2.4 Genomic alterations in gallbladder BiIN and in ICPN

In the nineties Wistuba et al. detected a high incidence of TP53 overexpression in immunohistochemistry in GBC and dysplastic lesions of the gallbladder that are compatible with the current definition of low and high grade BiIN (66) (Table 7). The overexpression of TP53 usually correlates with the presence of missense mutations in TP53 because the half-life of mutated and defective TP53 protein is increased, allowing it to be detected in immunohistochemistry, whereas the TP53 wild type protein is rapidly degraded in the nucleus (66). Thus, the presence of missense mutations in TP53 were suspected in gallbladder BiIN, while BiIN-derived intrahepatic cholangiocarcinomas seemed to involve Kras mutations..

Recently, Lin et al. studied the genomic landscape of precursor lesions in GBC and proposed new evolutionary trajectories from precursor lesions to cancer (67). (Table 7)

Biliary tract cancer	BiIN	ICPN
Gallbladder cancer	P53 overexpression TP53 mutations Mutations in B-catenin, ERBB3, ARID2 Amplification of MDM2, YEATS4, CDK4/6, ERBB3	Few data: KRAS mutations

Table 7. : Genomic alterations in gallbladder BiIN and ICPN

They selected 11 GBC patients presenting concurrent premalignant lesions described as low-grade and high-grade BiIN. These lesions were subjected to laser capture microdissection and whole-exome sequencing. Of note, they used a confusing nomenclature describing low-grade BiIN as gallbladder adenoma with low-grade dysplasia and it is not possible to assess which precursor lesions they have actually considered. Also, their introduction is not in line with the current knowledge in the field and several statements are not justified by the references. Still, their molecular analysis is pioneering and detected CTNNB1/ β -catenin and ARID2 mutations in 5 out of 11 patients while TP53 and ERBB3 mutations were detected in 3 out of 11 patients. These four genes were mutated also in precursor lesions. (Figure 14). The five patients whose GBC were mutated for β -catenin also

presented a β -catenin mutation in at least one of the considered precursor lesions. The reported mutations in β -catenin have shown their gain-of-function potential in hepatocellular tumors (hepatocellular adenomas and/or hepatocellular carcinomas) (68) and hepatocellular adenomas mutated for β -catenin present a malignant potential. These data support the hypothesis that β -catenin mutations are drivers in GBC initiation. In contrast, ARID2 and ERBB3 mutations are usually detected either in GBC or in the reported precursor lesions. TP53 missense and non-sense mutations were detected in 3 precursor lesions out of the 11 patients (67).

Brägelmann et al. studied copy number variations in 10 gallbladder high grade BILINs and identified gains of MDM2, YEATS4, CDK4, CDK6 and ERBB3 in these dysplastic lesions (Table 1). MDM2 and YEATS4 are two negative regulators of TP53 (69) (Table 7).

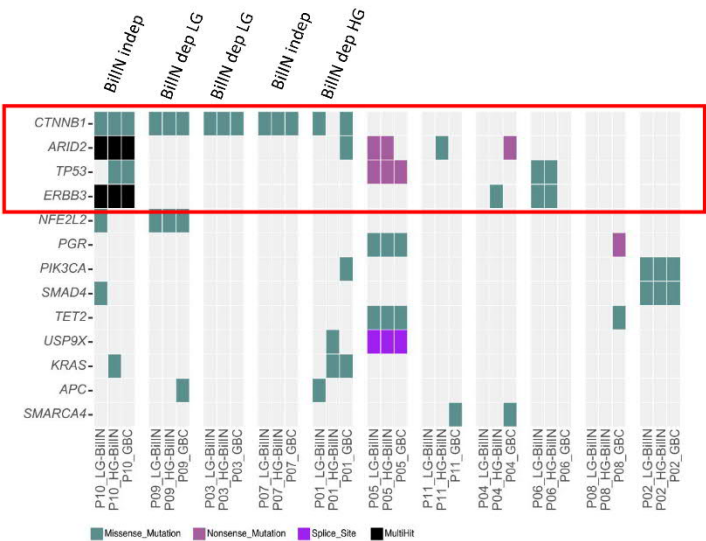


Figure 14: Mutated genes in gallbladder BILIN and GBC following Lin and coworkers. Adapted from Lin, NatCommunications, 2021

Data concerning the mutational landscape of ICPN are limited due to the rarity of the lesion and to the variability of definitions and terminologies used to describe it. KRAS mutations appear to be more frequent in ICPN than in gallbladder BILIN (10) (Table 7)

Lin et al. inferred different evolutionary trajectories between the lesions called low grade BiIN, high grade BiIN and GBC, based on the analysis of the single nucleotide variation and loss-of-heterozygosity patterns between different lesions from the same patient. This analysis of concurrent lesions within the same patient uncovers two distinct but coexisting evolutionary models of GBC, that they called BiIN-dependent and BiIN-independent models (Figure 15). In the BiIN-dependent model, GBC is more related from a genomic point of view to high grade BiIN or low grade BiIN depending on the patient considered. In contrast, the BiIN-independent model relies on the fact that some GBC included in the cohort differ from both low and high grade BiIN from a genomic point of view.

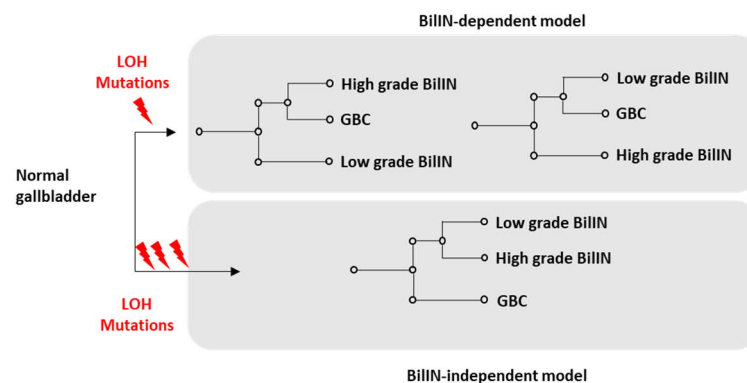


Figure 15: Distinct evolutionary trajectories of GBC. Adapted from Lin et al. Nat Commun 2021;12:4753.

These models challenge the accepted dysplasia-carcinoma sequence in gallbladder carcinogenesis. Indeed, following the BiIN-dependent model, GBC could originate from both low grade or high grade BiIN and not only from the high grade BiIN. The BiIN-independent model goes one step further because it considers that low and high grade BiINs evolve in parallel with GBC and that there is no filiation between BiIN and GBC. That means that BiIN are not preneoplastic/precursor per se in that context.

Moreover, following Lin et al.'s analysis, two factors influence the cell fate decision and the subsequent trajectory of the epithelial cell, namely the number of somatic mutations and the proportion of loss-of-heterozygosity events occurring at the first steps of tumor development in the putative common ancestor. Both were indeed higher in the BillIN-independent model. This suggests that extensive genomic alterations occurring early during the process are able to induce malignant transformation *de novo*, whereas when the mutational load is lower at the beginning, epithelial cells need a stepwise progression to acquire full malignant potential.

Transcriptomic events occurring specifically in BillIN or IPNB/ ICPN are poorly known, since most RNA sequencing analyses concern full-blown biliary tract cancers. Recently, two distinct high-throughput analyses provide additional information in the field of gallbladder carcinogenesis.

I.2.2.5 Transcriptomic events in gallbladder BillIN

One approach to better understand molecular mechanisms driving the onset of the tumor development is to compare the methylome and transcriptomic profile of invasive cancer with the reported precursor lesions.

Brägelmann et al. were the first to suggest the activation of the Hedgehog pathway in gallbladder high grade BillIN and GBC (69). They performed an epigenome-wide analysis of the methylation changes in the course of GBC tumorigenesis from gallstone disease, dysplasia and GBC. The name dysplasia refers here to BillIN. Of note, while Lin and coworkers studied concurrent lesions from the same patient, this study included different lesions from different patients : 88 Chilean patients (33 gallstone disease, 15 low-grade dysplasia, 10 high grade dysplasia and 30 GBC). It revealed a global methylation increase from gallstone disease to GBC with hypermethylation predominantly affecting the promoter regions of genes and leading to their transcriptional silencing. When the global methylation status of the lesions was summarized on a PCA plot (principal

component analysis), 3 clusters appeared. Methylome profiles of gallstone disease and low-grade dysplasia samples were comparable and these lesions created a unique cluster which differed from GBC. The group of high grade dysplasia fell just in between these two clusters.(Figure 16). For the analysis, the authors create two

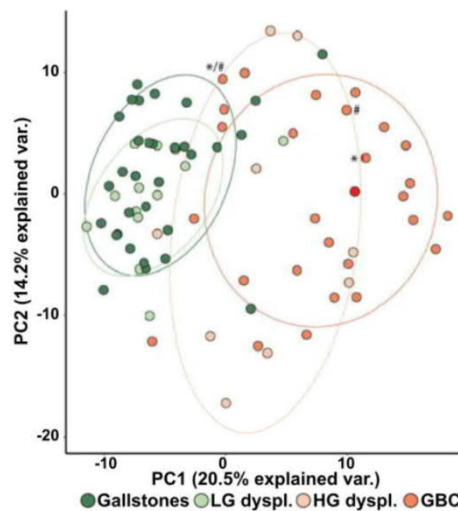


Figure 16: Methylation profiles of gallbladders affected with gallstone disease, low grade dysplasia (LG) , high grade dysplasia (HG) or GBC. Principal component analysis (PCA) based on the normalized methylation values for the 10,000 most variable markers.From Bragelmann, Hepatology, 2020.

groups based on this PCA plot: one group includes gallstone disease and low grade dysplasia samples, the other group includes high-risk dysplasia and GBC samples.

Some of the top differentially methylated genes between these two groups were ZIC 1 and ZIC4 which are known for their inhibitory effect on Hedgehog signaling, suggesting an impact of the activation of the Hedgehog pathway in gallbladder carcinogenesis (69) (Table 8). Other candidate genes were RUNX3 and WIF1. RUNX3 showed the most significant promoter hypermethylation difference between the two groups mentioned before (gallstone disease plus low grade dysplasia versus high-grade dysplasia plus GBC) and is a known tumor suppressor gene in gastric cancer (70). RUNX3 hypermethylation in high risk dysplasia and GBC leading to its silencing could thus stimulate tumor initiation. WIF1 (Wnt inhibitory factor 1) promoter showed a monotonous increase of methylation along the

sequence gallstone disease-low grade dysplasia-high grade dysplasia-GBC, supporting the stimulatory role of the Wnt/ β -Catenin pathway in the first steps of tumor development (69).

For these reasons, I think that activations of the Hedgehog and/or the Wnt/ β -catenin pathways eventually associated with an inhibition of RUNX3 could be determinant in the first steps of gallbladder carcinogenesis. (Table 8).

Potential drivers in GBC initiation	Arguments	Reference
Hedgehog pathway	Hypermethylation of inhibitors (ZIC1 and ZIC4) in BiliIN HG and GBC	Bragelmann, 2020
RUNX3 inhibition/ loss-of-function	Hypermethylation of RUNX3 in BiliIN HG and GBC	Bragelmann, 2020
Wnt/ β -Catenin	Hypermethylation of Wnt inhibitors (WIF-1) during gallbladder carcinogenesis	Bragelmann, 2020
EIF2 pathway	Enriched along the sequence cancer-premalignant lesions-normal cells	Zhang, 2021
CDK1	Upregulated in cancer cells versus premalignant state	Zhang, 2021
CXCL5	Upregulated in tipping point versus normal cells	Zhang, 2021
C-MYC	Upregulated in tipping point versus normal cells	Zhang, 2021

Table 8: Potential drivers in GBC initiation.

Additional RNA sequencing analysis of GBC cells and normal cells supported the involvement of EIF2, CDK1, CXCL5 and C-MYC. (Table 8.) in GBC initiation. Zhang and co-workers proposed a pseudo-time analysis in the course of gallbladder carcinogenesis, inferred from RNA single cell sequencing of GBC and normal epithelium within the same patients. (65). Regarding the epithelial compartment, this analysis revealed four clusters in epithelial cells. Epithelial subtype 1 was enriched in adjacent normal tissues which should represent normal epithelial cells. In contrast, epithelial cell types 2, 3 and 4 were mainly found in cancers, subtypes 2 and 3 in adenocarcinomas and subtype 4 in adenosquamous carcinoma, a rare variant of GBC. In an attempt to model the first steps of tumorigenesis in GBC, a pseudo-time analysis was performed using the epithelial cell subtype 1 to model normal cells and epithelial cell subtype 2 to represent cancer cells. Subtype 4 was not included in the trajectory analysis because it represents a rare histological variant of GBC and probably reflects the presence of squamous cells. Epithelial subtype 2 was chosen to represent cancer cells because it showed an enrichment of cell division pathways and regulation of mitosis, whereas epithelial cell subtype

3 was enriched in immune related processes. A small subpopulation of 50 cells was identified in the transition between normal and cancer tissues and contained a mixed population made of epithelial cells subtypes 1 and 2. These cells are referred by the authors as the tipping point and could represent a premalignant state (Figure 17).

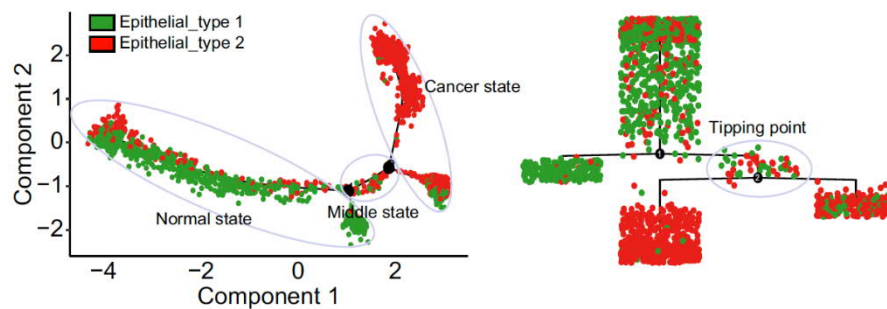


Figure 17. Proposed tumorigenic pathway of epithelial cell types in gallbladder cancer. Pseudotime analysis identifies three states. From Zhang et al. J Hepatol 2021; 75:1128-1141

The most enriched signaling pathway in the sequence epithelial cell subtype 1-tipping point-epithelial cell subtype 2 is the EIF2 pathway. This suggests the involvement of EIF2, a translation initiation factor, in the first steps of tumor development in gallbladder cancer. CXCL5 and C-MYC were described by the authors as upregulated in the tipping point versus epithelial cell subtype 1. Although this is unclear following the published data if these upregulations are statistically significant, this could make sense from a biological point of view because CXCL5 is a chemokine and inflammatory conditions are known to promote biliary tract cancers and c-MYC is a known oncogene (71). Finally, CDK1 (cyclin dependent kinase 1) was also upregulated between epithelial cell subtype 2 and the tipping point.

To conclude, these sequencing analyses provide clues for understanding the molecular mechanisms driving epithelial cell fate decisions during gallbladder carcinogenesis. As mentioned above, we aimed to focus on the changes concerning the epithelial compartment rather than the tumor microenvironment in GBC and CCA. Although extensive data confirm the crosstalk between epithelial cells and

stromal/immune cells in cancer, little attention was paid to potential interactions with the stroma at the first steps of tumor development, i.e. in precursor lesions. Still, the role of stromal cells in cancer initiation must not be underestimated. Indeed, stromal cells can produce matrix metalloproteinases (MMP) able to perforate the basement membrane, acting as a physical barrier that prevents spreading of dysplastic cells in the micro-environment. (72). Moreover, Glentis and co-workers showed in an in vitro model of cancer invasion that CAF contributed to the first steps of invasion by remodeling the basement membrane using MMP-independent mechanisms. In this model, CAFs pull and push on the basement membrane by contracting along it and eventually break it. They also produce bundles of extracellular matrix along the basement membrane. These bundles modify the physical properties of the membrane and make it more fragile (72).

I.2.3 Putative cell(s) of origin of biliary tract cancers

It is tempting to speculate that the different subtypes of BTC reflect distinct cells of origin, as described by Banales and coworkers in their review.(1) about cholangiocarcinomas (Figure 18).

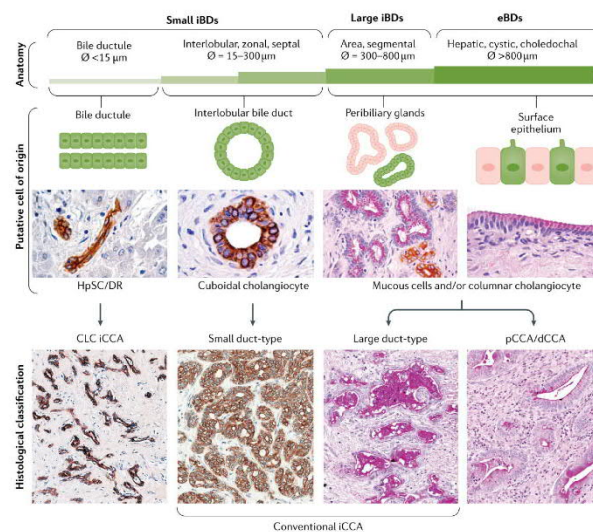


Figure 18. Putative cell of origin of cholangiocarcinoma. From Banales, Nature reviews, 2020. CLC= cholangiolocellular carcinoma. HpSC= hepatic progenitor cell. DR= ductular reaction.

The main risks factors of eCCA (primary sclerosing cholangitis, flukes infection, lithiasis, smoking and choledocal cysts) target the cholangiocytes lining the bile ducts (referred here as surface epithelium) and eCCA are lined by columnar and mucin-producing cells, as do normal extrahepatic cholangiocytes. Although it is impossible to show it in humans, extrahepatic cholangiocytes lining the bile ducts could thus represent the cell of origin of eCCA. However, the extrahepatic bile ducts also contain peribiliary glands (as do hilar bile ducts and large intrahepatic bile ducts in humans). These seromucinous glands were claimed to contain multipotent stem cells that could be at the origin of CCA because cells isolated from peribiliary glands were able to differentiate into cholangiocytes, hepatocytes and

pancreatic islets cells under specific conditions in culture (73) . We can debate about the scientific arguments used to claim that there is a stem compartment in the peribiliary glands. Still, evidences from transgenic mouse models propose, in a well argued and balanced way, that eCCA could originate from peribiliary glands because the mutations required to induce cholangiocarcinogenesis (Kras^{G12D} mutation associated with TGFβR2 and E-Cadherin deletions) induced the elimination of the cells when targeting lining cholangiocytes while mutated cells remained in the peribiliary glands (23). Of note, peribiliary glands are described along the cystic duct but it seems unlikely that GBC originates from peribiliary glands given that GBC usually locates in the fundus of the gallbladder.

Because intrahepatic large bile ducts are also lined by columnar cholangiocytes and contain peribiliary glands, the same rationale could be applied to iCCA, especially to the large duct type iCCAs that stained cytoplasmatic for EMA (MUC1) as do large bile duct cholangiocytes (Figure 19).(7)

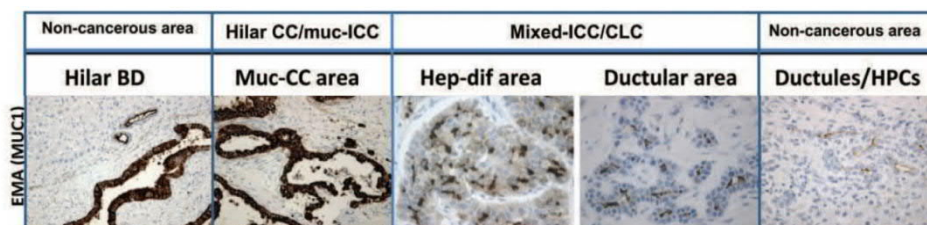


Figure 19: Histological diversity of iCCA. Adapted from Komuta, Hepatology, 2012. BD= bile duct. CC= cholangiocarcinoma. Muc-ICC= mucin producing intrahepatic cholangiocarcinoma. Mixed-ICC =mixed intrahepatic cholangiocarcinoma (combined hepatocarcinoma-cholangiocarcinoma). CLC= cholangiocarcinoma. HPCs: hepatic progenitor cells.

The recognition of the small duct type iCCA as a separate entity raised new questions concerning the cell at the origin of these tumors. Small duct type iCCA are made of cuboidal cholangiocytes or ductular reaction-like structures in the cholangiolocellular subtype. Both are characterized by an apical staining for EMA (MUC1) and reminiscent of interlobular bile ducts and ductules , respectively that

also stain for EMA at the apical membrane (Figures 18 and 19). Thus, it is possible that small duct type iCCA arise from small duct cholangiocytes, i.e. interlobular bile ducts and bile ductules.

Another option is that a subset of iCCA actually derives from transdifferentiation of hepatocytes. Indeed, iCCA also arises in non-biliary cirrhosis and hepatitis B and C infections, two conditions primarily targeting the hepatocytes. Moreover, mice experiments show that the induction of the Notch pathway in the hepatocytes generates iCCA. (19). Unfortunately, it is not possible to assess if these iCCA fit with small duct type iCCA described in humans. Moreover, epidemiological studies usually do not mention the histological subtype of tumor considered. In my view, it is thus not possible to assess if the iCCA arising in patients affected with non-biliary cirrhosis or hepatitis are actually small-duct type iCCA.

Distinct cell types could therefore be at the origin of BTCs but how these distinct origins influence the phenotype and the behaviour of these tumors is unclear. Better standardization of studies and improved integration of all epidemiological, molecular and histological data should enable us to better understand these differences in the future. Along the same lines, although precursor lesions have been described in BTCs, the mechanisms leading to their development and the forces driving their transformation or senescence obviously deserve more attention.

II. Goals of the research project

The aim of the thesis is to uncover molecular mechanisms promoting tumor initiation in biliary tract cancers. We specifically studied iCCA and GBC focusing on their precursor lesions and their evolution toward malignancy. To address these issues we used transgenic mouse models recapitulating as faithfully as possible human pathology, and spatial transcriptomic analyses of human samples.

The work has been organized in two sections.

First, I characterized the histology of precursor and malignant lesions in a transgenic mouse model of iCCA that combines an inducible and cholangiocyte-specific activating mutation of KRAS with chronic diet-induced cholangitis.

Second, I tried to investigate the first steps of the carcinogenesis in gallbladder adenocarcinoma by developing new transgenic mouse models that combine the most frequent mutations described in the literature. In parallel, I analyzed human precursor lesions of GBC using a spatial transcriptomic approach and I tried to recapitulate the first steps of tumor development using non-tumoral (human) gallbladder organoids.

Contributions to the work

The development of the mouse model of iCCA was ongoing when I joined the laboratory. The transgenic mouse model had been optimized and was known to induce cancers. However, a thorough pathological analysis was missing. I analyzed the phenotype of the transgenic mice and described the occurrence of adenocarcinomas compatible with large duct type iCCA associated with IPNB lesions. I performed macroscopic analyzes to exclude metastatic adenocarcinoma to the liver and characterized the subtype of iCCA. I suggested that iCCA likely originated from IPNB rather than ductular proliferations in that model and I looked for histological signs supporting this hypothesis. I described transitions between IPNB and glandular proliferations which I considered and defined as early stage iCCA.

The microdissections of the different subtypes of lesions and the RNA extractions were performed by Mickaël Di-Luoffo. Analysis of the sequencing data was realized by Axelle Lorient, Mickaël Di-Luoffo and Pr Lemaigre. I was mainly involved in the immunohistochemical analyzes needed to address the two hypotheses we had chosen to explore (SOX17 and TNS4). Transfection experiments and migration or proliferation assays were performed by Mickaël Di-Luoffo and Sabine Cordi. I performed the IL-33 experiments in collaboration with Florence Lamoline (Figure Suppl 6C).

When we decided to attempt building a mouse model of GBC, I analyzed the literature and selected 4 target genes (P53, KRAS, ERBB3 and APOBEC3B) because they were recurrently mutated in GBC. Some of them were also reported to be mutated early in the carcinogenic process. The generation of ERBB3^{V104M} required cloning experiments that I performed with the help of Mohamad Assi. Younes Achouri performed the CrispRCas9 experiment to clone the sequence of interest in the Rosa²⁶ locus. I analyzed the phenotype of these mice in the gallbladder, the extrahepatic bile ducts and the liver. Because they were histologically normal, I checked the expression of the transgenes and looked for explanations for its variability.

In parallel, I developed a spatial transcriptomic analysis of BILIN and GBC from human samples. I was inspired by a series of GBC cases that I reviewed during my assistantship. I studied the different methods of spatial transcriptomic that would be suitable to analyze archival material, and investigated in detail the histology of the 15 GBC cases that were available. We complied with the ethical rules before using human materials and subsequently selected the regions of interest. Attempts to extract DNA in sufficient amount for targeted sequencing were unsuccessful.

The spatial transcriptomic data collection was performed by Nanostring, under my guidance to select the appropriate regions of interest. Axelle Lorient and Laurent Gatto performed the quality control and classification of the data. With their help, I established working hypotheses to address the function of genes in tumor progression.

To test these hypotheses, we needed a model of normal cells and we derived non-tumoral organoids from non-tumoral human gallbladders. I was

trained to generate, culture and characterize these organoids by Fatima Manzano. I characterized these organoids by immunolabellings, functional assays, DNA sequencing and karyotyping (in collaboration with Sébastien Toffoli). We tested the tumorigenic properties of the non-tumoral organoids alone or after a pretreatment with a cocktail of estrogens, bile acids, increased concentrations EGF and TGF β inhibitors subcutaneously in immunodeficient mice. None of these conditions gave rise to tumor. Because the levels of expression of SEMA4A fitted very well with our 2 distinct models of gallbladder carcinogenesis and because I thought that SEMA4A could be measured in blood, I found an antibody known to block SEMA4A induced signaling. I added this antibody in the culture medium of the organoids, which allowed us to detect pseudostratification of the epithelium reminiscent of BillIN.

Because the number of in vitro tests available to study organoids was limited, Fatima advised me to tackle the question the other way around and to use EGI-1 cells treated with SEMA4 rh protein. She performed the transwell and clonogenic assays showing a reduced migration and clonogenicity under SEMA4A rh protein. I detected an increase in tumor growth in xenograft mouse models treated with the SEMA4A blocking antibody, which was independently confirmed by Fatima.

III. Results

The results achieved in the two sections of the work are presented as summarized in the plan below. We refer to the original papers included in the manuscript for the published results.

- Intrahepatic cholangiocarcinoma

Di-Luoffo M, Pirenne S, Saandi T, Lorient A, Gérard C, Dauguet N, Manzano-Núñez F, Alves Souza Carvalhais N, Lamoline F, Konobrocka K, De Greef V, Komuta M, Halder G, Jacquemin P, Lemaigre FP. A mouse model of cholangiocarcinoma uncovers a role for Tensin-4 in tumor progression. *Hepatology* 74, 1445-1460, 2021.

In this work, I performed the pathological analyses of Figures 1, 2 and 5 and I contributed to the Supplementary Figure 6C. I realized and analyzed SOX17 and TNS4 immunolabelling.

- Gallbladder cancer
 - Mouse models of gallbladder carcinogenesis
 - Spatial transcriptomics of human gallbladder cancer and biliary intraepithelial neoplasia

III.1 Intrahepatic cholangiocarcinoma

A Mouse Model of Cholangiocarcinoma Uncovers a Role for Tensin-4 in Tumor Progression

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BACKGROUND AND AIMS: Earlier diagnosis and treatment of intrahepatic cholangiocarcinoma (iCCA) are necessary to improve therapy, yet limited information is available about initiation and evolution of iCCA precursor lesions. Therefore, there is a need to identify mechanisms driving formation of precancerous lesions and their progression toward invasive tumors using experimental models that faithfully recapitulate human tumorigenesis.

APPROACH AND RESULTS: To this end, we generated a mouse model which combines cholangiocyte-specific expression of *Kras*^{G12D} with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet-induced inflammation to mimic iCCA development in patients with cholangitis. Histological and transcriptomic analyses of the mouse precursor lesions and iCCA were performed and compared with human analyses. The function of genes overexpressed during tumorigenesis was investigated in human cell lines. We found that mice expressing *Kras*^{G12D} in cholangiocytes and fed a DDC diet developed cholangitis, ductular proliferations, intraductal papillary neoplasms of bile ducts (IPNBs), and, eventually, iCCAs. The histology of mouse and human IPNBs was similar, and mouse iCCAs displayed histological characteristics of human mucin-producing, large-duct-type iCCA. Signaling pathways activated in human iCCA were also activated in mice. The identification of transition zones between IPNB and iCCA on tissue sections, combined with RNA-sequencing analyses of the lesions supported

that iCCAs derive from IPNBs. We further provide evidence that tensin-4 (*TNS4*), which is stimulated by *KRAS*^{G12D} and SRY-related HMG box transcription factor 17, promotes tumor progression.

CONCLUSIONS: We developed a mouse model that faithfully recapitulates human iCCA tumorigenesis and identified a gene cascade which involves TNS4 and promotes tumor progression. (HEPATOLOGY 2021;74:1445-1460).

Prognosis of intrahepatic cholangiocarcinoma (iCCA) remains dismal. Given that this results, in part, from delayed diagnosis, there is a need to understand the mechanisms that drive development of precancerous lesions and their progression toward invasive tumors. A range of perturbed signaling pathways and genomic alterations is associated with late-stage cholangiocarcinoma (CCA), but there is still limited knowledge about how such pathways and mutations evolve during tumorigenesis.⁽¹⁻³⁾ Moreover, our understanding of this process is further complicated by the observation that iCCA can arise from several cell types.⁽⁴⁻¹⁰⁾

Histopathological studies identified two iCCA subtypes in humans. Small duct subtype is located at the

Abbreviations: AKT, protein kinase B; Aspa, aspartoacylase; BillIN, biliary intraepithelial neoplasia; CCA, cholangiocarcinoma; cDNA, complementary DNA; CK19, cytokeratin 19; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; EGFR, epidermal growth factor receptor; EpCAM, epithelial cell adhesion molecule; ERK, extracellular regulated kinase; ERBB2, v-erb b2 erythroblastic leukemia viral oncogene homolog 2; cYFP, enhanced yellow fluorescent protein; HES1, hairy and enhancer of split-1; HNF4, hepatocyte nuclear factor 4; iCCA, intrahepatic cholangiocarcinoma; IPNB, intraductal papillary neoplasm of bile ducts; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; NICD, intracellular domain of Notch1; p-AKT, phosphorylated AKT; p-ERK, phosphorylated ERK; PI3K, phosphoinositide-3-kinase; p-SMAD2, phosphorylated SMAD2; SMAD2, small mothers against decapentaplegic 2; SOX9, SRY-related HMG box transcription factor 9; SOX17, SRY-related HMG box transcription factor 17; TCGA, The Cancer Genome Atlas; TGFβ, transforming growth factor beta; TNS4, tensin-4.

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Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31834/supinfo.

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liver periphery and presents as tubular-type adenocarcinoma with cuboidal cells and ductular component without mucin production. The large duct subtype is found near the hilum and exhibits a tubular pattern with mucin-producing columnar cells.⁽¹¹⁾ Histopathological studies classified biliary intraepithelial neoplasia (BillIN), intraductal tubulopapillary neoplasms, and intraductal papillary neoplasms of bile ducts (IPNBs) as noninvasive precursor lesions.⁽¹²⁻¹⁴⁾ Whereas BillINs develop in large bile ducts and present as flat or micropapillary dysplasia of the bile duct epithelium, IPNBs are macroscopic lesions of the large ducts characterized by exophytic papillary structures with fibrovascular core and prominent intraductal growth.^(15,16) von Meyenburg complexes are irregular ductal structures surrounded by dense stroma. These complexes are benign, but were reported to evolve toward iCCA in rare instances.

Gain-of-function mutations of the small GTPase v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) are detected in iCCA at a frequency of 15%-25% and are associated with a significant decrease of survival.⁽¹⁷⁻¹⁹⁾ Importantly, *KRAS* mutations were detected in BillINs and IPNBs in 25%-50% of cases, suggesting that such mutations may contribute to initiate tumorigenesis.⁽²⁰⁻²²⁾ Chronic inflammation is a known risk factor of iCCA. Patients with primary sclerosing cholangitis are at risk for iCCA, and molecular classification of late-stage CCAs underscored the importance of inflammatory gene expression in iCCA pathogeny.⁽²³⁻²⁵⁾

Several animal models of intra- and extrahepatic CCA have been designed.^(26,27) However, limited attention was paid to tumor initiation and progression, and in several instances, genetic alterations were induced at the prenatal stage or simultaneously in hepatocytes and cholangiocytes, unlike in humans. Here, we generated an original mouse model of iCCA tumorigenesis in which expression of oncogenic *Kras*^{G12D} is specifically induced in cholangiocytes and is combined with diet-induced inflammation that mimics chronic cholangitis. Mice developed IPNB similar to those in humans, and our data support that those lesions evolve toward iCCA. We also identified a gene-regulatory pathway that is activated during tumorigenesis and which promotes cell proliferation and migration.

Materials and Methods

ANIMALS

Animals were monitored daily and received humane care according to the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences), to the Directive 2010/63/EU of the European Parliament; and to the Belgian law of May 29, 2013 on protection of animals used for experimentation, updated on September 7, 2017 by the Brussels Region. Protocols were approved by the Animal Welfare Committee of the Université catholique de Louvain with license number 2018/UCL/

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*These authors contributed equally to this work.

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Potential conflict of interest: Nothing to report.

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MD/014, and by the Institutional Ethical Commission for Animal Research at Katholieke Universiteit Leuven. Osteopontin-iCreERT² (Opn-Cre^{ER}), loxP-stop-loxP-Kras^{G12D/+} (LSL-Kras^{G12D/+}), and Rosa26R^{eYFP} mice were described⁽²⁸⁻³⁰⁾ and bred in a CD1-enriched background. For Cre-LoxP recombination, 6-week-old mice were injected intraperitoneally three times at a 2-day interval with tamoxifen (T5648; Sigma-Aldrich, Bornem, Belgium) dissolved in corn oil (30 mg/mL, at a dose of 150 mg/kg). Mice were fed a standard diet or a diet containing 3,5-diethoxycarbonyl-1,4 dihydrocolidine 0.1% (DDC; 137030; Sigma-Aldrich) and monitored for signs of illness as described in the Results section and in Supporting Fig. S1.

Animal Numbers

Wild-type mice or tamoxifen-treated Opn-Cre^{ER} mice (devoid of mutated loxP-stop-loxP-Kras^{G12D} allele) were indistinguishable at the histological level and were used as controls. The number of mice used in the study was as follows: wild-type Kras + DDC: 20; Kras^{G12D}: 30; and Kras^{G12D} + DDC: 60.

LASER CAPTURE MICRODISSECTION, RNA EXTRACTION, AND SEQUENCING

Livers were collected, embedded in Optical Cutting Temperature, frozen in liquid nitrogen, and stored at -80°C. Microscope support glass, membrane slides, and tools were pretreated to remove RNase (RNaseZAP; Sigma-Aldrich). Ten-micrometer sections were cut using Cryostar NX70 (ThermoFisherScientific) at -25°C for knife/blade temperature and -13°C for specimen temperature. Liver sections were deposited on slides adapted for laser capture microdissection (LCM; SuperFrost Plus [25 × 75 × 1 mm]; VWR, Leuven, Belgium) and stored at -80°C inside plastic containers with silica granules to prevent tissue hydration. Within 12 hours, liver sections were microdissected using a Leica LMD7000 microscope (GIGA, Liège, Belgium), and RNA was extracted with an RNAqueous-Micro total RNA isolation AM1931 Kit (ThermoFisherScientific). RNA-sequencing was performed by the Next Generation Sequencing Service (NGS Genewiz, South Plainfield, NJ). Quality of samples was controlled by NanoDrop (NanoDrop 2000 [ThermoFisherScientific] and Qubit fluorometry

[Invitrogen, Carlsbad, CA]) for measuring concentration and calculation of RNA integrity number (Agilent 2100 Bioanalyzer System). RNA was amplified using the SMARTer Universal Low Input RNA Kit for sequencing (Takara, Mountain View, CA). Ribosomal RNA (rRNA) was depleted using the RiboGone-Mammalian rRNA removal kit (Takara), and complementary DNA (cDNA) libraries were generated using the TruSeq total stranded RNA-sequencing kit (Illumina, San Diego, CA). cDNA libraries were sequenced using Illumina HiSeq 2 × 150-bp configuration with estimated data output ≥350 M raw paired-end reads per lane. Data are stored under Gene Expression Omnibus (GEO) accession number GSE141511.

Other Materials and Methods are available in the Supporting Information.

Results

CHOLANGIOCYTE-SPECIFIC EXPRESSION OF Kras^{G12D} COMBINED WITH DDC-INDUCED CHOLANGITIS CAUSES DEVELOPMENT OF IPNB AND ICCA

To recapitulate tumorigenesis in human iCCA, we generated a mouse model, which combines cholangiocyte-specific Kras^{G12D} expression with chronic cholangitis. Opn-Cre^{ER} mice, which express tamoxifen-inducible Cre^{ER} recombinase specifically in cholangiocytes, were mated with LSL-Kras^{G12D/+} mice, which contain an inducible mutant Kras^{G12D} allele.⁽²⁸⁻³⁰⁾ Mice also contained a Rosa26R^{eYFP} allele. Kras^{G12D} expression was induced by administration of tamoxifen to 6-week-old mice. Mice were then fed a normal diet for 4 weeks to enable washout of tamoxifen. Thereafter, chronic cholangitis was induced by feeding the mice a DDC diet.⁽³¹⁾ DDC feeding was interrupted after 2 weeks because of weight loss. Following 1 week of weight recovery under normal diet, DDC diet was resumed, and livers were collected after 8 and 18 weeks of cholangitis and at several time points between 21 and 31 weeks of cholangitis (Fig. 1A). During that period, mice accepted the DDC diet; the experiments were interrupted when animals showed signs of severe illness and had reached humane endpoints. These conditions did not allow to reveal a significant impact of Kras^{G12D} on the survival of DDC-treated animals (Supporting Fig. S1A-C).

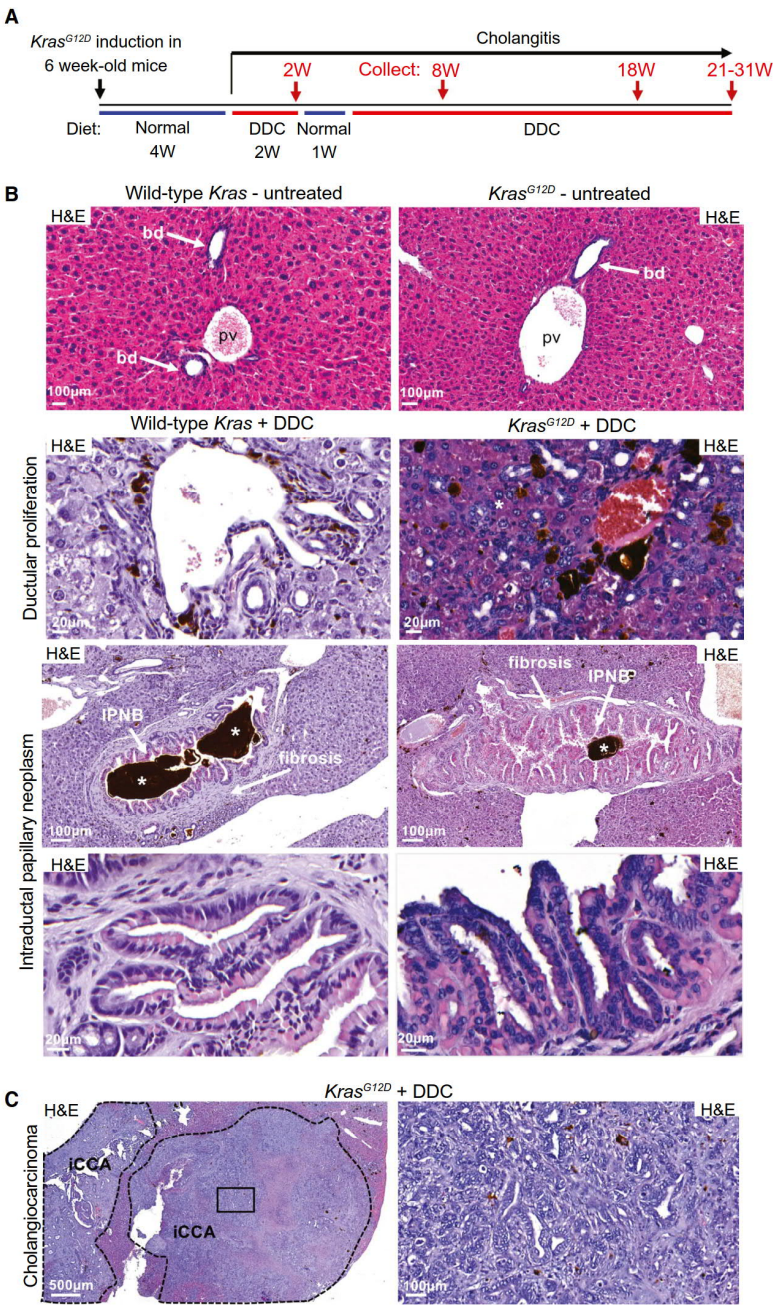


FIG. 1. Biliary lesions develop following diet-induced chronic cholangitis and cholangiocyte-specific expression of *Kras*^{G12D}. (A) Timing of *Kras*^{G12D} expression and of DDC-induced cholangitis. (B) Opn-Cre^{ER}/LSL-*Kras*^{G12D} mice expressing *Kras*^{G12D} following tamoxifen administration (*Kras*^{G12D}) do not show any lesion, whereas mice treated with DDC for 18 weeks in the absence (wild-type *Kras* + DDC) or presence of *KRAS*^{G12D} (*Kras*^{G12D} + DDC) develop ductular proliferations and IPNB with fibrous thickening of the bile duct wall. (C) iCCAs are detected only in *Kras*^{G12D} + DDC mice and starting at 21 weeks of DDC treatment. Boxed region in the left panel is magnified in the right panel. *Porphyrin plug. Abbreviations: bd, bile duct; pv, portal vein; W, weeks.

Expression of *Kras*^{G12D} in the absence of DDC did not affect bile duct morphology. Administration of DDC for 8 weeks to wild-type mice induced accumulation of porphyrin plugs, ductular proliferation, as well as emerging papillary lesions and periductal fibrosis of large ducts. In contrast, expression of *Kras*^{G12D} in mice receiving an 8-week DDC treatment was associated with large duct lesions, which displayed papillary proliferation, similar to IPNB (Supporting Fig. S1D).

At 18 weeks of DDC feeding, large duct lesions in mice expressing wild-type *Kras* had evolved to IPNB, and ductular proliferations were more extended (Fig. 1B). Expression of *Kras*^{G12D} in the absence of DDC still did not affect the histology of the biliary epithelium (Figs. 1B and 2A). At 18 weeks of DDC treatment, expression of *Kras*^{G12D} was associated with IPNBs, which did not significantly differ from those monitored in mice expressing wild-type *Kras* (Fig. 1B).

Starting at 21 weeks of the DDC diet, iCCAs were observed, but only in mice combining DDC-induced cholangitis and cholangiocyte-specific *Kras*^{G12D} expression (Fig. 1C). At that stage, iCCAs had developed in one third of cases (12 of 36 males; 9 of 24 females) and showed tubular structures with desmoplastic changes typical of human CCA (Fig. 1C). iCCAs were proliferative, as illustrated by Ki67 staining, and expressed cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EPCAM); they also expressed enhanced yellow fluorescent protein (eYFP), supporting their biliary origin (Fig. 2A). IPNBs maintained their biliary fate, as evidenced by the expression of SRY-related HMG box transcription factor 9 (SOX9), and lack of hepatocyte nuclear factor 4 (HNF4). iCCA cells expressed SOX9. A subset of iCCA cells were HNF4 positive (Fig. 2A). Given the high cholangiocyte specificity of Opn-Cre^{ER}, it is unlikely that these HNF4-positive cells derive from hepatocytes; rather, HNF4 expression likely reflects dedifferentiation. The epithelium of the iCCA was columnar to cuboidal and was mucin positive, as shown by alcian blue staining (Fig. 2B). This phenotype is similar to the human mucin-producing large-duct-type iCCA. Some tumors appeared less desmoplastic (Fig. 2B). Interestingly, adjacent to large ducts, we often found areas of glandular

proliferation with expansive growth pattern and cytoneuclear atypia, which we qualified as early stage of invasive cancer. Such lesions were detected starting ~18 weeks of DDC treatment (Supporting Fig. S1C). Importantly, focal transitions between IPNB and early invasive cancer were detected, suggesting that IPNB evolved to iCCA (arrows in Fig. 2C).

We concluded that mice develop IPNB and iCCA, which faithfully recapitulate the histology of their human counterparts.

SIGNALING PATHWAYS DRIVING ICCA DEVELOPMENT IN HUMANS ARE ACTIVATED IN MICE

To investigate the signaling pathways often perturbed in human iCCA, we first evaluated mitogen-activated protein kinase activity and looked at phosphorylated extracellular-regulated kinase (p-ERK).⁽³²⁾ p-ERK was detected in cholangiocytes following *Kras*^{G12D} induction or DDC treatment, and in iCCA, but not in untreated normal cholangiocytes (Fig. 3A). Second, phosphoinositide-3-kinase (PI3K) signaling⁽³²⁾ was activated in IPNB cells of DDC-treated mice expressing wild-type *Kras*, and in iCCA of DDC-treated mice expressing mutant *Kras*^{G12D}, as evidenced by staining with antiphosphorylated protein kinase B (AKT) serine/threonine kinase (phosphorylated AKT [p-AKT]; Fig. 3A). Third, we also detected accumulation of hairy and enhancer of split-1 (HES1)⁽³³⁾ in nuclei of iCCA, indicating that the Notch pathway was stimulated (Fig. 3A). Phospho- (p-SMAD2) small mothers against decapentaplegic 2 (SMAD2), a marker of transforming growth factor beta (TGFβ) activity, was detected at high levels only in IPNB, not in other conditions. Finally, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2)/human epidermal growth factor receptor 2⁽³⁴⁾ was detected in all conditions. Microdissection of IPNBs and iCCAs from tissues of mice expressing mutant *Kras*^{G12D} and treated for 25 weeks with DDC, followed by western blotting, indicated that the amount of ERBB2 protein was higher in iCCA than in IPNB, and that ERBB2

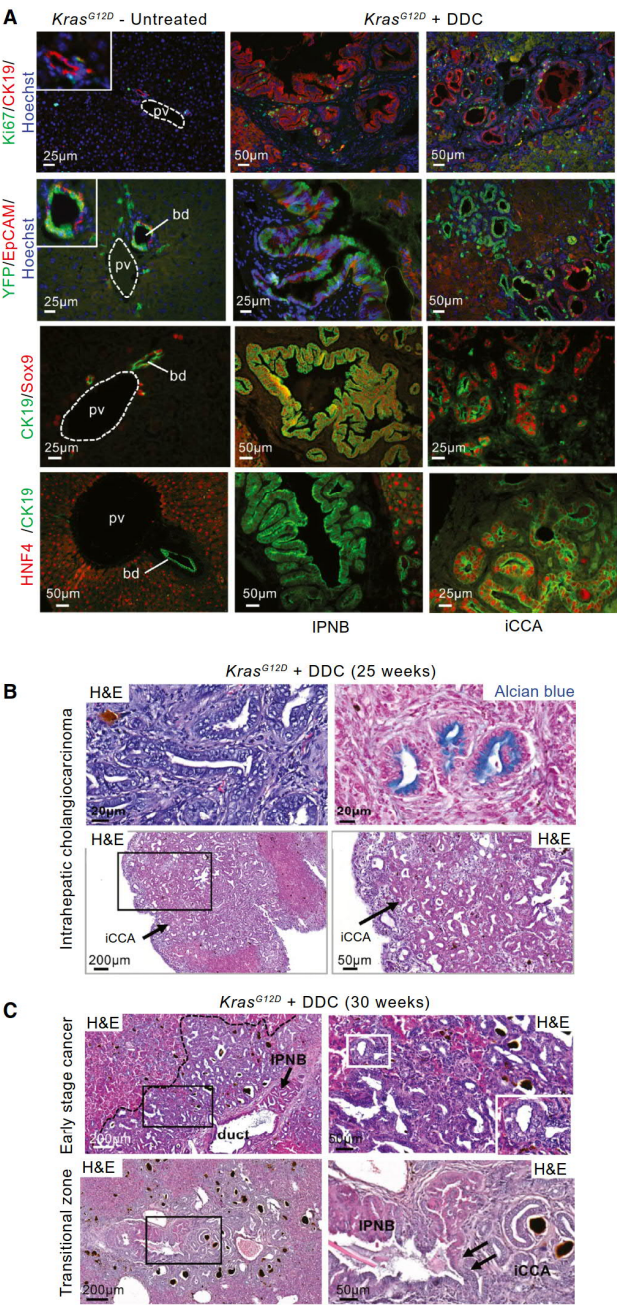


FIG. 2. Histomorphology of biliary lesions in mice expressing *Kras*^{G12D} and fed a cholangitis-inducing diet. (A) In Opn-Cre^{ER}/LSL-Kras^{G12D}/Rosa26^{eYFP} mice fed a DDC diet for 21 weeks (*Kras*^{G12D} + DDC), cells of IPNB and iCCA proliferate, as shown by Ki67 staining; they also express CK19 and EpCAM; eYFP expression demonstrates their cholangiocyte origin. IPNB and iCCA express SOX9, but not HNF4. In iCCA, a subset of cells express detectable HNF4. (B) iCCA in *Kras*^{G12D} + DDC mice shows glandular structures displaying columnar to cuboidal morphology and fibroinflammatory stroma. iCCA cells express mucins, as evidenced by alcian blue staining. (C) A transition zone between IPNB and the early stage of invasive iCCA (black arrows) is detected in *Kras*^{G12D} + DDC mice. Inset in panel C (right) points to nuclear atypia. Boxed regions in left panels are magnified in right panels. Abbreviations: bd, bile duct; pv, portal vein.

was active, as evidenced by detection of phosphorylated ERBB2^{Y1248} (Fig. 3B). Also, RT-qPCR analysis of total RNA from normal purified cholangiocytes and from microdissected IPNB and iCCA showed higher expression of epidermal growth factor receptor (EGFR) in the lesions (Fig. 3B).

We concluded that MAPK, AKT, Notch, TGFβ, and ERBB signaling was activated during tumorigenesis in our mouse model, like in human iCCA.

IPNB AND ICCA DISPLAY SIMILAR GENE EXPRESSION PROFILES AND OVEREXPRESS TNS4

To gain insight into the mechanisms of tumor progression, we investigated the gene expression profile of ductular proliferations, IPNBs, and iCCAs in DDC-treated mice expressing mutant *Kras*^{G12D}. The three types of lesions coexisted in livers treated for 25 weeks with DDC and were isolated at that stage. Microdissected iCCAs displayed the histology of the early stage of invasive cancer, as illustrated in Fig. 2B. Microdissection was performed by LCM followed by RNA-sequencing. Principal component analysis (PCA) of the transcriptomic data revealed that ductular proliferations clustered separately from iCCAs and IPNBs, which clustered more closely (Fig. 4A). Combined with the location of iCCAs near large ducts containing IPNB and the existence of transitional zones between IPNB and iCCA (Fig. 2C), the closely related gene expression pattern suggests that iCCAs originate from IPNB. Nineteen genes were found to be differentially expressed between IPNB and iCCA (fold-change, >2; adjusted *P* value, <0.05), of which 10 were overexpressed (Fig. 4B). Except for aspartoacylase (*Aspa*)/Acy2 gene aminoacylase 2 (*Acy2*), these 10 genes were also overexpressed in human iCCA versus nontumor tissue (The Cancer Genome Atlas cohort [TCGA]; *n* = 34; <http://firebrowse.org>) and/or GEO cohort GSE119336 (*n* = 15),⁽³⁵⁾ supporting

the relevance of the mouse data with human data (Fig. 4C).

The focal adhesion protein, tensin-4 (TNS4; also called C-terminal tensin-like protein), attracted our attention because it is up-regulated by extracellular-regulated kinase (ERK) signaling and promotes tumor progression in HCC.⁽³⁶⁾ RNA-sequencing showed significant overexpression of *Tns4* in iCCA as compared to IPNB (Fig. 4B). RT-PCR showed higher mean values of *Tns4* in iCCA as compared to IPNB, and in *Kras*^{G12D}-expressing cholangiocytes as compared to wild type, albeit with variability among samples. Western blotting of microdissected IPNBs and iCCAs confirmed that TNS4 is significantly higher in iCCA compared to IPNB (Fig. 5A). Immunostainings confirmed the expression of TNS4 in mouse bile ducts, IPNB, and iCCA (Fig. 5B). Also in humans, TNS4 was up-regulated in iCCA as compared to controls (Fig. 5C).

TNS4 expression was also investigated in iCCA derived from hepatocytes. We overexpressed AKT and the intracellular domain of Notch1 (NICD) in hepatocytes of adult mice by hydrodynamic tail vein injection of plasmid DNA as described.⁽⁸⁾ These mice developed iCCAs 6 weeks after injection (Fig. 5D). Similarly, expression of AKT, NICD, and KRAS^{G12V} also resulted in development of iCCA 3 weeks after injection. Immunostaining revealed strong expression of TNS4 in iCCA cells (Fig. 5D), indicating that TNS4 is expressed both in cholangiocyte-derived (Opn-Cre^{ER}/LSL-Kras^{G12D} + DDC model) and in hepatocyte-derived iCCA (AKT + NICD and AKT + NICD + KRAS^{G12V} models).

TNS4 IS STIMULATED BY KRAS SIGNALING AND STIMULATES MIGRATION AND PROLIFERATION

We next explored whether KRAS activated expression of TNS4. Given that EGI-1 cells constitutively

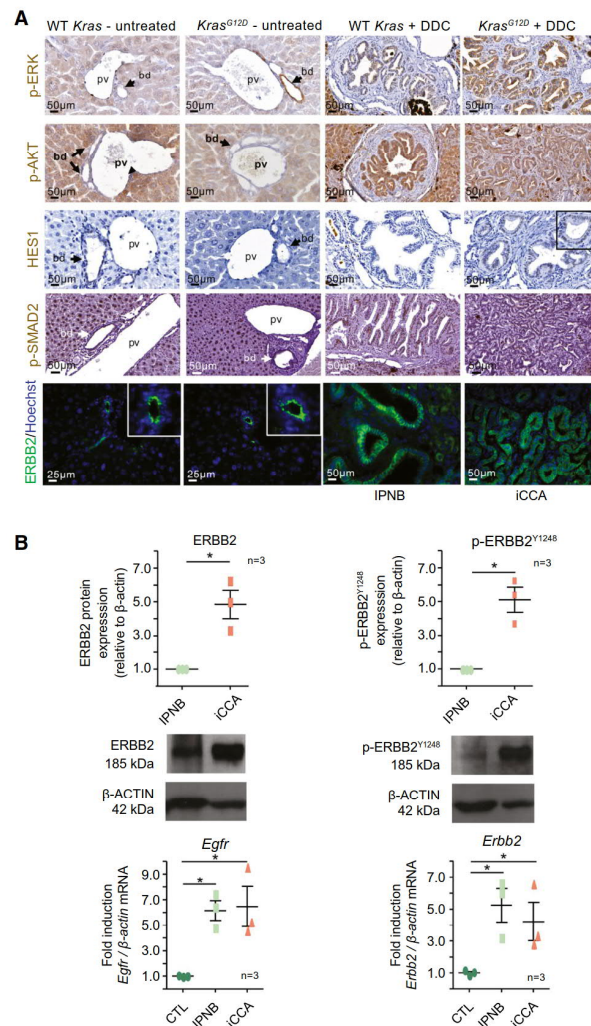


FIG. 3. Signaling pathways activated during tumor progression. (A) p-ERK staining is detected in cholangiocytes following *Kras*^{G12D} expression, in DDC-induced IPNB and *Kras*^{G12D} + DDC-induced iCCA. p-AKT is detected in IPNB and iCCA, whereas HES1 is detected only in iCCA. p-SMAD2 is detected only in IPNB. ERBB2 is detected in biliary cells in all conditions. (B) Levels of ERBB2 are higher in iCCA than in IPNB, and ERBB2 protein is active as evidenced by detection of p-ERBB2^{Y1248}. Expression of *Egfr* and *ErbB2* mRNA was higher in IPNB and iCCA than in normal purified cholangiocytes. Dot plots show means ± SEM. **P* < 0.05. Abbreviations: CTL, control; WT, wild type.

express *KRAS*^{G12D}, we transiently transfected NMC cells with *Kras*^{G12D} to verify whether it stimulates *Tns4* in cholangiocytes; this was the case (Fig. 6A).

Given that TNS4 is known to promote hepatocarcinoma cell migration,⁽³⁶⁾ we verified whether this holds true in biliary cells. We generated EGI-1 and

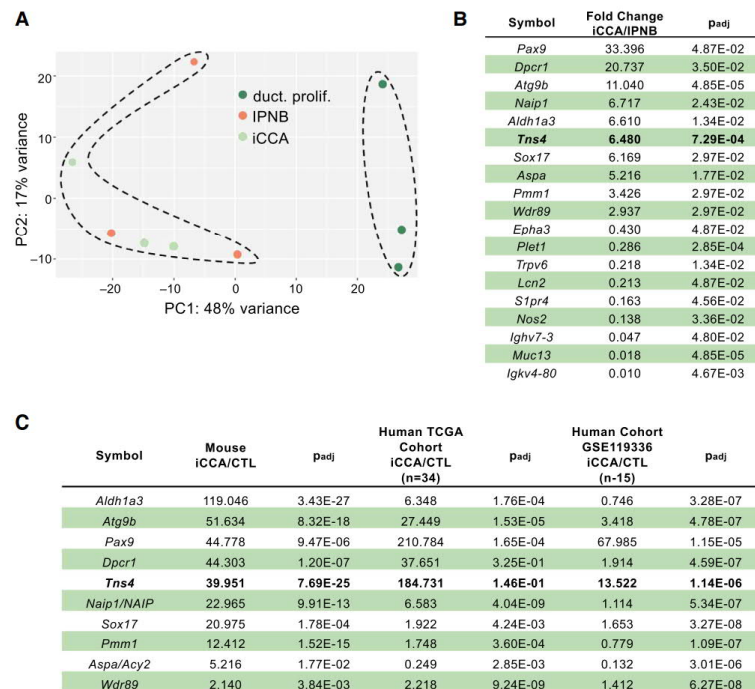


FIG. 4. Differentially expressed genes between IPNB and iCCA. (A) PCA of RNA-sequencing data of ductular proliferations, IPNB, and iCCA. Lesions were microdissected from tamoxifen-treated Opn-Cre^{ER}/LSL-Kras^{G12D}/Rosa26R^{YFP} mice fed a DDC diet for 25 weeks. (B) Table of the 19 genes differentially expressed between IPNB and iCCA. (C) Genes that are overexpressed in murine iCCA versus IPNB are also overexpressed in murine iCCA versus control cholangiocytes. Control cholangiocytes are FACS purified from tamoxifen-treated Opn-Cre^{ER}/Rosa26R^{YFP} mice. Most of these genes are overexpressed in human iCCA versus control tissue. Two human iCCA cohorts are compared with mouse iCCA. Abbreviations: *Aldh1a3*, aldehyde dehydrogenase 1 family member A3; *Atg9b*, autophagy-related 9B; CTL, control; *Dpcr1*, diffuse panbronchiolitis critical region 1; duct. prolif., ductular proliferation; *Epha3*, EPH receptor A3; FACS, fluorescence-activated cell sorting; *Ighv7-3*, immunoglobulin heavy variable 7-3; *Igkv4-80*, immunoglobulin kappa variable 4-80; *Lcn2*, lipocalin-2; *Muc13*, mucin 13, cell surface associated; NAIP, NLR family apoptosis inhibitory protein; *Naip1*, NLR family apoptosis inhibitory protein 1; *Nos2*, nitric oxide synthase 2; *Pax9*, paired box gene 9; PC1, principal component 1; PC2, principal component 2; *Plet1*, placenta expressed transcript 1; *Pmm1*, phosphomannomutase 1; *S1pr4*, sphingosine-1-phosphate receptor 4; *Trpv6*, transient receptor potential cation channel subfamily V member 6; *Wdr89*, WD repeat-containing protein 89.

NMC clones that stably overexpress doxycycline-inducible TNS4 (Fig. 6B), and found that induction of TNS4 in both cell types accelerated cell migration in a wound-healing assay (Fig. 6D). The assay lasted for 8 hours, that is, below the duration of doubling time. More important, doxycycline-mediated the increase of TNS4 stimulated proliferation of EGI-1 cells, as shown by crystal violet staining (Fig. 6E). We also used CRISPR-Cas9 (CRISPR-associated protein 9) to generate EGI-1 cell knockdown for

TNS4 (Fig. 6C and Supporting Fig. S3). *In vitro* knockdown cells displayed reduced proliferation as evidenced by the longer time required to reach confluence (Fig. 6E).

To test whether TNS4 was sufficient to stimulate growth *in vivo*, we overexpressed TNS4 in EGI-1 cells and subcutaneously injected them into NOD-scid gamma (NSG) mice. The results showed that TNS4 accelerates tumor growth *in vivo* (Fig. 6F). Conversely, subcutaneous injection of 10⁶ EGI-1 or

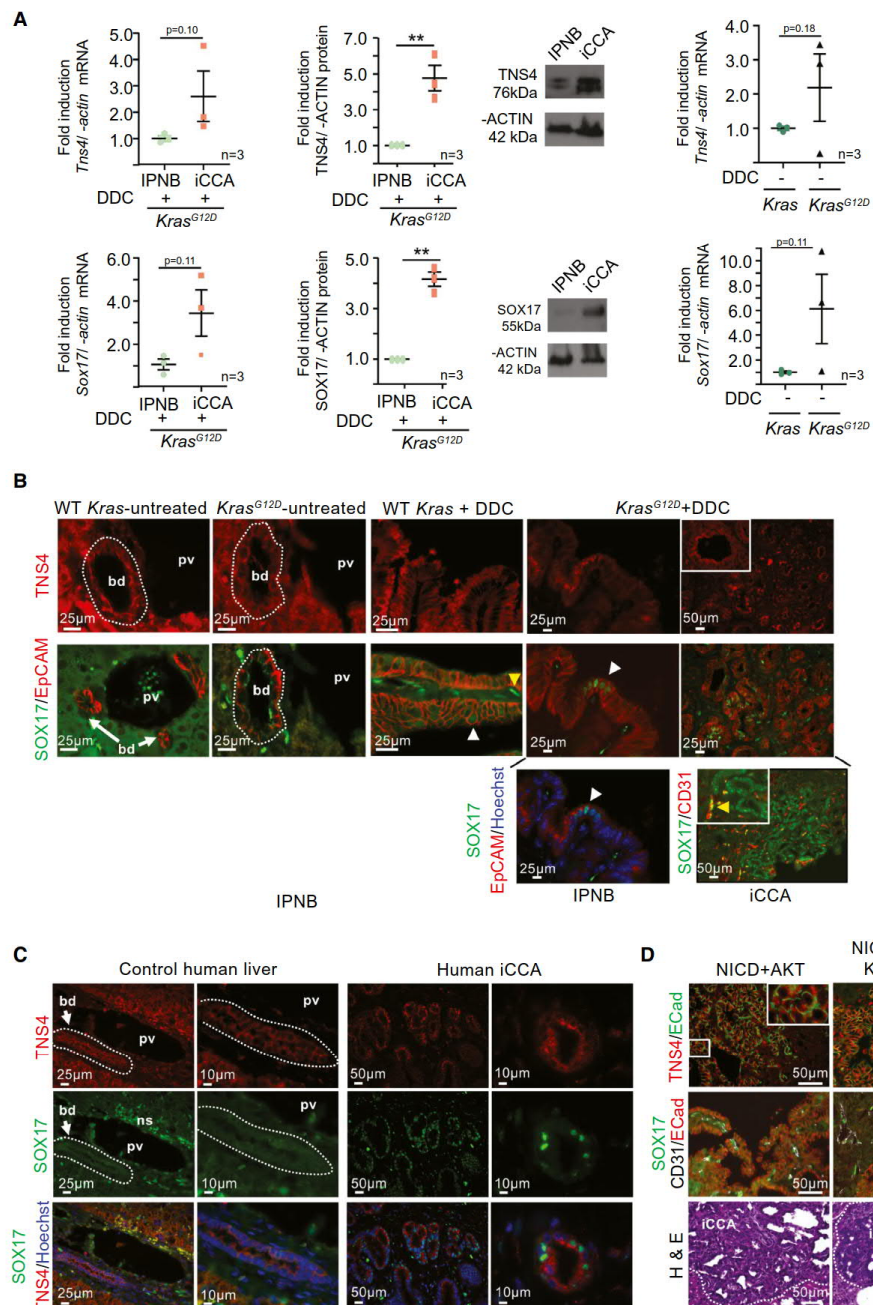


FIG. 5. TNS4 and SOX17 expression in mouse and human iCCA. (A) RT-PCR shows higher mean values of *Tns4* and *Sox17* in iCCA as compared to IPNB, and in *Kras*^{G12D}-expressing cholangiocytes as compared to wild type, albeit with variability among samples. Western blotting of microdissected IPNBs and iCCAs show significant up-regulation of TNS4 and SOX17 proteins in iCCA as compared to IPNB. IPNB and iCCA were microdissected from mice expressing *Kras*^{G12D} and fed a DDC diet for 25 weeks. Cholangiocytes from mice fed a normal diet were FACS sorted from tamoxifen-treated Opn-Cre^{ER}/Rosa26R^{eYFP} or Opn-Cre^{ER}/LSL-Kras^{G12D}/Rosa26R^{eYFP} mice, as indicated. Data in dot plots are means $\pm$ SEM. ***P* < 0.01. (B) Expression of TNS4 is detected in biliary cells from normal mouse ducts, IPNB, and iCCA. SOX17 is detected in DDC-induced IPNB and iCCA, not in cholangiocytes from untreated mice. (C) TNS4 and SOX17 are detected in human iCCA. Dotted lines delineate bile ducts; white arrowheads, SOX17-expressing biliary cells; yellow arrowheads, SOX17-expressing CD31-positive endothelial cells. (D) TNS4 is detected in iCCA cells, which developed in mice overexpressing AKT and NICD or AKT, NICD, and *Kras*^{G12V} in hepatocytes. SOX17 expression was restricted to endothelial cells. Insets represent high magnifications. Abbreviations: bd, bile duct; ECad, E-cadherin; FACS, fluorescence-activated cell sorting; ns, nonspecific labeling; pv, portal vein.

TNS4 knockdown EGI-1 cells resulted in strongly reduced tumor development as compared to control EGI-1 cells (Fig. 6F).

SRY-RELATED HMG BOX TRANSCRIPTION FACTOR 17 CONTROLS EXPRESSION OF TNS4

Our data above show that the transcription factor, SRY-related HMG box transcription factor 17 (SOX17), is among the 10 genes that are overexpressed in iCCA as compared to IPNB in mice expressing *Kras*^{G12D} and treated for 25 weeks with DDC (Fig. 4B). RT-PCR data showed that mean expression of *Sox17* was increased in iCCA as compared to IPNB, albeit with variability among samples. Western blotting of microdissected IPNBs and iCCAs confirmed the results and showed significant up-regulation of SOX17 protein in iCCA as compared to IPNB (Fig. 5A). By immunostaining on liver sections, SOX17 was not detected in normal cholangiocytes or in *Kras*^{G12D}-expressing cholangiocytes. In IPNBs from DDC-treated animals expressing wild-type *Kras*, a low number of cells expressed SOX17 at levels slightly higher than background. In contrast, in DDC-treated animals expressing *Kras*^{G12D}, patches of epithelial cells in IPNB and iCCA showed significant levels of nuclear SOX17 (Fig. 5B). These immunostaining data were confirmed by western blotting (Fig. 5A). SOX17 was also detected in endothelial CD31-positive cells, as expected.⁽³⁷⁾ Importantly, human livers displayed a similar profile with no detectable SOX17 in normal cholangiocytes, but with obvious expression in iCCA cells (Fig. 5C).

TNS4 was detected at the cell membrane in all tested conditions. SOX17-positive cells coexpressed TNS4 in iCCA (Fig. 5C). In addition, *SOX17* and

TNS4 mRNA expression levels were correlated in human iCCA (Fig. 7A and Supporting Fig. S4). Moreover, given that KRAS is known to stimulate SOX17 expression,⁽³⁸⁾ we tested the possibility that SOX17 activates TNS4. We generated EGI-1 clones that stably overexpress doxycycline-inducible SOX17 (Fig. 7B), and found that SOX17 stimulated TNS4 and *KRAS* expression (Fig. 7C). Stimulation of TNS4 is likely indirect given that screening 28 kilobase pairs of the *TNS4* gene (−2,500 to +25,500) for SOX17 binding sites using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) or AliBaba2 (<http://gene-regulation.com/pub/programs/alibaba2/index.html>) revealed no SOX17 consensus binding site. We also transfected EGI-1 cells with a TNS4-luciferase construct comprising 2,197 bp (−2,148 to +49) of the human *TNS4* promoter subcloned upstream of the firefly luciferase cDNA in pGL3-basic. This construct generated a 22-fold higher luciferase activity than pGL3-basic, demonstrating *TNS4* promoter activity. However, it was not stimulated following cotransfection of a SOX17 expression vector (fold change = 0.79 ± 0.27 SD; *P* = 0.38; *n* = 3). In addition, *SOX17* expression was activated by *Kras*^{G12D} as shown in NMC cells transiently transfected with a *Kras*^{G12D} expression vector (Fig. 6A). Finally, in EGI-1 cells overexpressing TNS4 under control of doxycycline, *KRAS* was slightly induced (Fig. 7C). Together, the data suggest the existence of a KRAS-SOX17-TNS4 cascade with positive feedbacks (Fig. 7C).

Discussion

Mouse models of CCA often induce genetic modifications at prenatal stages, or simultaneously in

multiple liver cell types, or in several organs.^(26,27) We selected Opn-Cre^{ER} for its high efficiency and cholangiocyte specificity to induce expression of *Kras*^{G12D}. Indeed, earlier work showed that Opn-Cre^{ER} triggers recombination of a reporter in up to 99.9% of cholangiocytes and in only 0.0074% hepatocytes; stellate

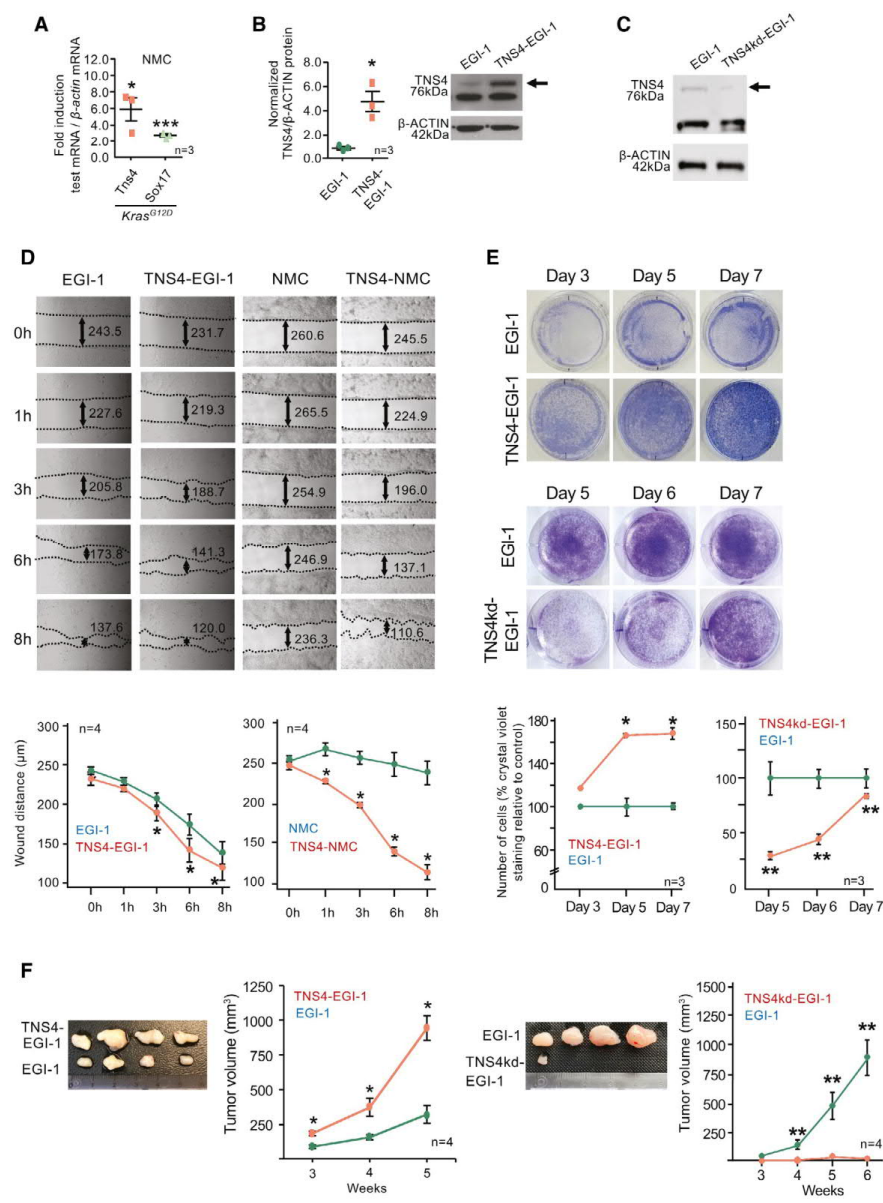


FIG. 6. TNS4 is stimulated by KRAS^{G12D} and promotes cell migration and tumor growth. (A) Transient transfection of *Kras*^{G12D} stimulates *Tns4* and *Sox17* expression in NMC cells. (B) Overexpression of TNS4 in EGI-1 cells stably expressing doxycycline-inducible TNS4 (TNS4-EGI-1), as compared to doxycycline-treated EGI-1 stably transfected with pSBtet-GN vector (EGI-1). Data in (B) and (C) represent fold inductions relative to control $\pm$ SEM. * $P < 0.05$. (C) Reduced expression of TNS4 in EGI-1 cells after CRISPR-Cas9-mediated targeting of the *TNS4* gene. Details are in Supporting Fig. S3. (D) Doxycycline-induced TNS4 accelerates migration of EGI-1 and NMC cells in a wound-healing assay. At each time point, the width of the wound is indicated as mean $\pm$ SEM (μ m) of three widths measured on six pictures covering the entire wound. (E) Doxycycline-induced TNS4 stimulates proliferation of EGI-1 cells; 16×10^4 cells were plated at day 1 (upper panels). Cells illustrated were counted after crystal violet staining (lower graphs). Data in graphs are means $\pm$ SEM. (F) Doxycycline-induced TNS4 stimulates tumor growth *in vivo* after subcutaneous injection of 10^6 EGI-1 or TNS4-EGI-1 cells in NSG mice. In contrast, TNS4kd-EGI-1 cells display reduced tumorigenesis as compared to control EGI-1 cells after subcutaneous injection. Tumor growth was measured with a caliper, and tumors were collected 5–6 weeks after injection (lower graphs). Data in graphs are means $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$.

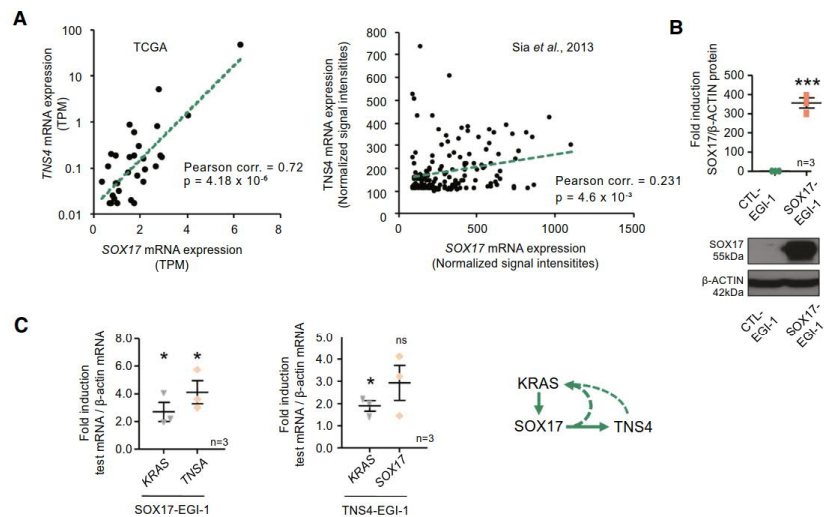


FIG. 7. A KRAS-stimulated cascade involving TNS4 and SOX17 is active during iCCA tumorigenesis. (A) Expression of *SOX17* and *TNS4* mRNA is correlated in human iCCA. Patient cohorts are from TCGA and Sia et al.⁽²⁵⁾ (B) Overexpression of SOX17 in EGI-1 cells stably expressing doxycycline-inducible SOX17 (SOX17-EGI-1), as compared to doxycycline-treated EGI-1 stably transfected with pSBtet-GN vector (EGI-1). (C) Left panel, SOX17 stimulates *KRAS* and *TNS4* in SOX17-EGI-1 cells. Right panel, TNS4 stimulates *KRAS* in TNS4-EGI-1. The structure of the KRAS-TNS4-SOX17 cascade is shown. Abbreviations: CTL, control; TPM, transcripts per million.

cells and macrophages did not show Opn-Cre^{ER} activity.^(28–39) Opn-Cre^{ER} activity in other organs is illustrated in Supporting Fig. S5. Tumors in extrahepatic ducts, gallbladder, and pancreas can show histological features comparable with iCCA. The absence of detectable tumors in these organs excluded that metastatic lesions were misidentified as iCCAs.

In our model, *Kras*^{G12D} is expressed from its endogenous locus. This was combined with chronic cholangitis to mimic tumor development in a context of

chronic inflammation, a major risk factor for iCCA. Tumorigenesis was associated with activation of ERK, AKT, NOTCH, and ERBB as in many human iCCAs. Also, mouse iCCAs displayed a tubular pattern with mucin-producing columnar cells, which is typical for human large-duct-type iCCA; intense desmoplasia was also detected. Therefore, our animal model displays many histological and biochemical features of human iCCA. No metastases were detected, yet their development may require prolonged DDC treatment,

but the deteriorating health prompted us to euthanize the animals. Moreover, the transcriptomic profile of IPNB and early stage of invasive iCCA, together with the histological identification of junctions between the two lesions, strongly suggest that iCCAs derive from IPNB. Interestingly, in humans, mutant *KRAS* is detected already in human low-grade IPNB, and tumor progression is associated with decreased TGF β signaling,⁽²²⁾ as in our model, which shows strong nuclear staining of p-SMAD2 in IPNB, but no detectable p-SMAD2 in iCCA. Activation of the Wnt pathway has been reported in a subset of human IPNB,⁽⁴⁰⁾ but we did not detect nuclear accumulation of β -catenin in our mice.

Fickert et al. described the inflammation occurring in DDC-treated livers.⁽³¹⁾ We monitored an increase in IL-33 expression in IPNB and further in iCCA (Supporting Fig. S6A). IL-33 is a mitogen which induces iCCA when combined with constitutively active AKT and Yes-associated protein (YAP) and with cholestasis.⁽⁴¹⁾ Figure 3A shows that AKT signaling was active during tumorigenesis in IPNB and iCCA, but the expression of Hippo-YAP signaling markers did not correlate with tumor progression (Supporting Fig. S6B). In our model, IL-33 injection to DDC-fed mice expressing *Kras*^{G12D} contributed to growth of the early-stage cancer lesions, but did not promote development toward a more advanced stage of iCCA (Supporting Fig. S6C).

Tensins are focal adhesion proteins involved in cell migration by bridging the extracellular matrix receptors and the actin skeleton. In hepatocellular cancer, TNS4 is activated by ERK and enhances cell motility.⁽³⁶⁾ In our mouse model, we detected TNS4 expression in normal cholangiocytes and monitored an increase in its expression during tumorigenesis. Moreover, TNS4 and SOX17 are colocalized in IPNB/iCCA SOX17-positive cholangiocytes, and in transfection experiments, we positioned TNS4 epistatically downstream of SOX17. TNS4 and SOX17 mRNA expression was positively correlated in human iCCA, as well as in breast carcinoma and prostate cancer, but not in other tumor types (Supporting Fig. S4B).

During tumorigenesis, we found that SOX17 becomes detectable at a low level in IPNB of DDC-treated mice expressing wild-type *Kras*. In iCCA, SOX17 was heterogeneously distributed, with large epithelial cell patches displaying high expression of SOX17, whereas other tumor cells lacked SOX17.

SOX17 was not detected in hepatocyte-derived iCCA cells (Fig. 5D). SOX17 was also strongly expressed in endothelial cells, both in cholangiocyte- and hepatocyte-derived iCCAs (Fig. 5B,D), meaning that care is needed to interpret transcriptomic and epigenomic analyses of SOX17 expression from sampled tumor tissues composed of multiple cell types. The higher level of SOX17 in microdissected iCCA as compared to IPNB likely reflects the higher number of cells expressing the gene in iCCA, and possibly the fact that SOX17-positive epithelial cells in iCCA express higher levels of SOX17 than in IPNB cells, as evidenced by more intense immunostaining in iCCA than in IPNB.

Furthermore, SOX17 is not expressed at the protein level in normal human or mouse intrahepatic cholangiocytes, but regulates development of the extrahepatic biliary tract and is detected in adult gallbladder epithelium.⁽⁴²⁾ Earlier work claims that SOX17 is detected in normal intrahepatic cholangiocytes.^(43,44) However, the immunostainings revealed only non-nuclear SOX17 in intrahepatic ducts, unlike in extrahepatic cholangiocytes where nuclear staining was obvious. In the absence of known nuclear-cytoplasmic shuttling, non-nuclear detection of SOX17 in intrahepatic ducts deserves re-evaluation. Interestingly, *Sox17* mRNA expression in wild-type or *Kras*^{G12D} mutant cholangiocytes does not correlate with SOX17 protein detection, indicating that this gene undergoes posttranscriptional regulation. SOX17 is regarded as a tumor suppressor in iCCA,^(44,45) and we confirmed that EGI-1 cells overexpressing SOX17 proliferate less well *in vivo* than control EGI-1 cells, as shown by Merino-Azpitarte et al. Importantly, given that TNS4 is expressed in several cells devoid of SOX17, the latter is not strictly required for TNS4 expression. However, our data showing that SOX17 can control TNS4 expression indicate that a global tumor-suppressor role of SOX17 does not exclude a contribution to tumor progression. In that context, we note that SOX17 was shown to be induced in precursor lesions of pancreatic ductal adenocarcinoma, and to cooperate with oncogenic KRAS^{G12D} to enhance transformation of normal pancreas.⁽³⁸⁾ Also, SOX17 is overexpressed in high-grade malignant ERBB2-transformed CCA cells as compared to less tumorigenic BDE-1 cells.⁽⁴⁶⁾ In conclusion, we have developed a mouse model that recapitulates

formation of iCCA precursor lesions and their evolution toward malignancy, much like in humans. Carcinogenesis is associated with up-regulation of TNS4, which promotes tumor progression and is regulated by KRAS and SOX17.

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Author Contributions:

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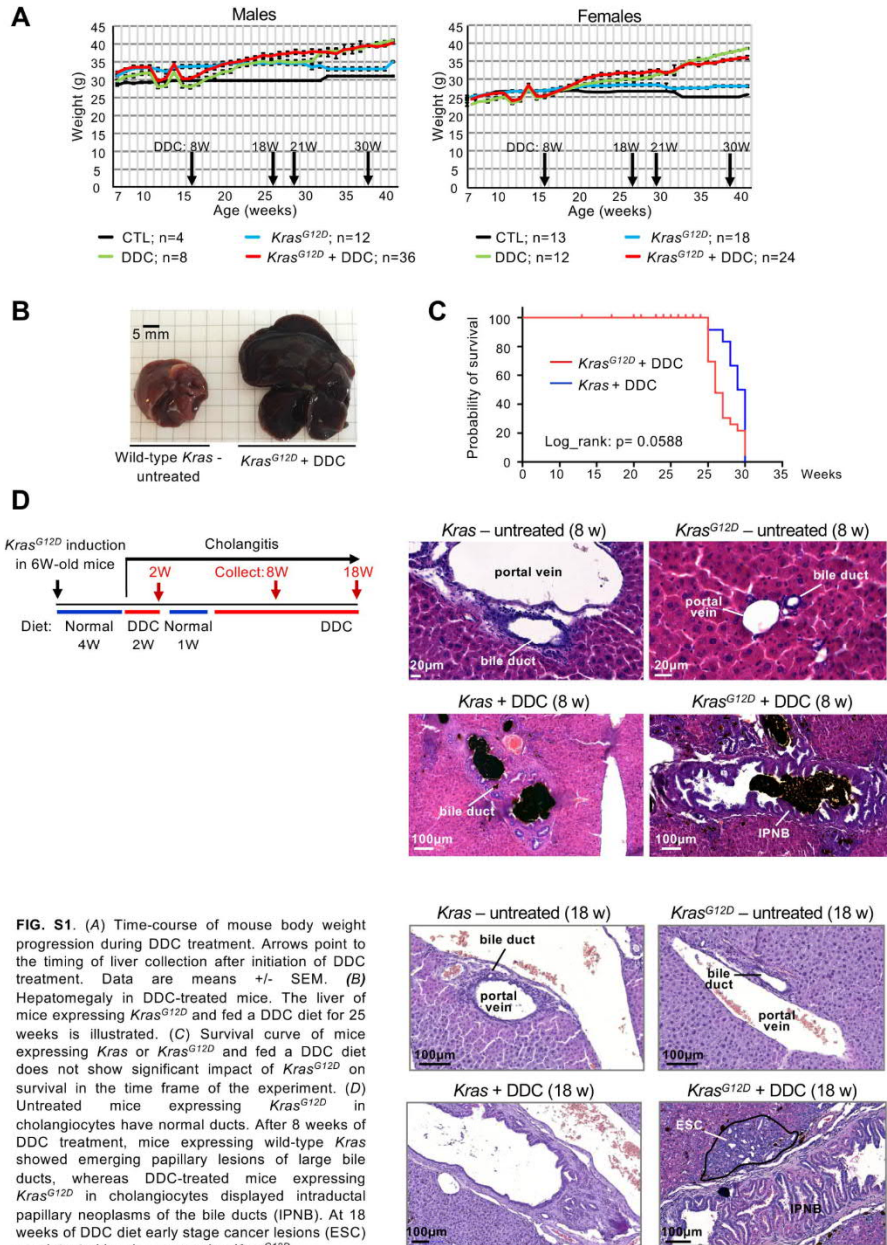
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Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31834/supinfo.



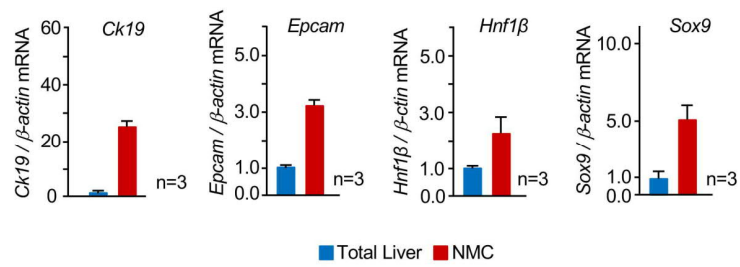


FIG. S2. NMC cells express cholangiocyte markers. The level of marker expression in cholangiocytes is compared with that in total adult mouse liver.

NB: FIG S3 is not shown

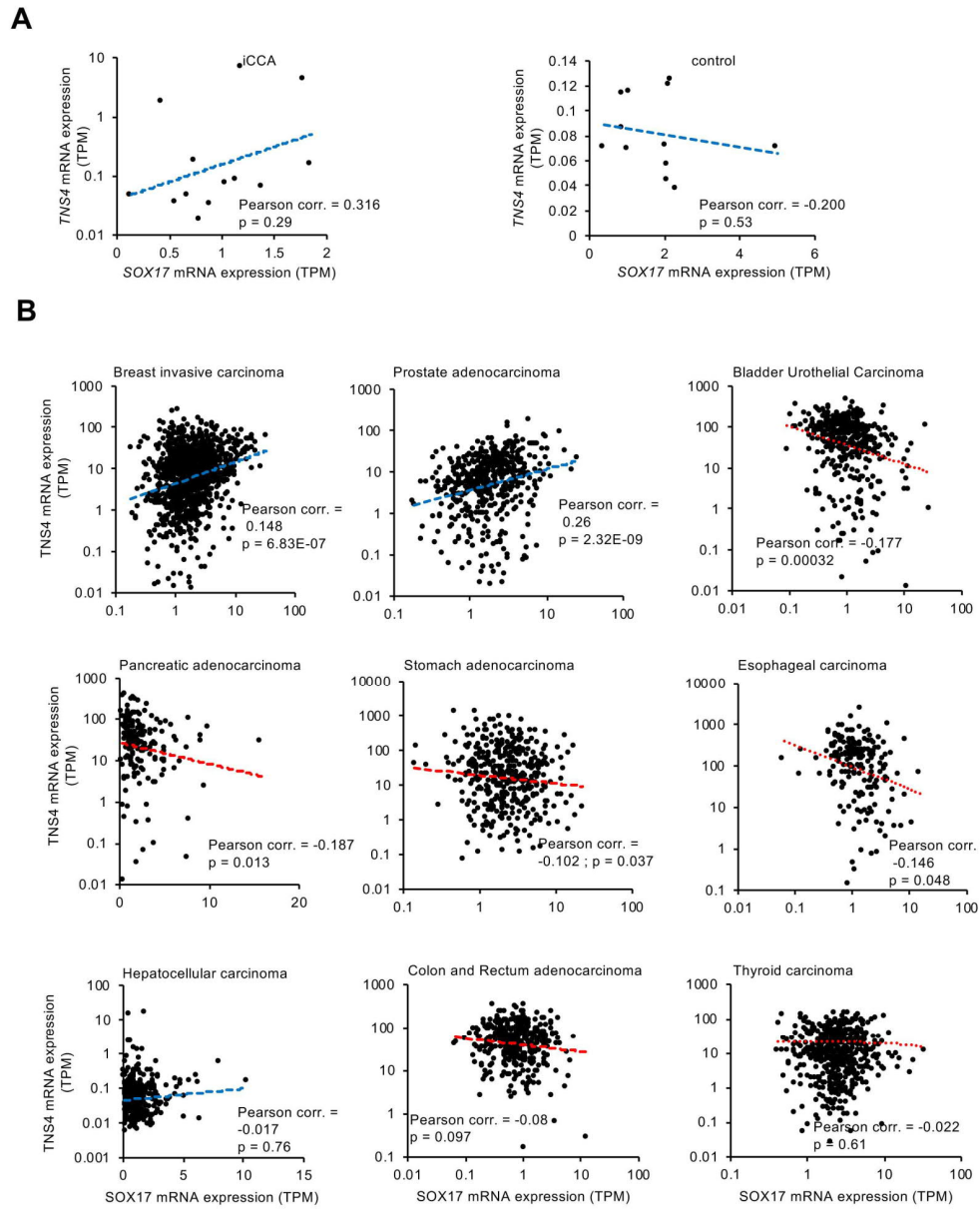


FIG. S4. (A) Correlation between *SOX17* and *TNS4* mRNA expression in the GSE119336 cohort. The correlation is not statistically significant in iCCA and the number of samples is low, but clearly differs from the controls. Control is non-tumor adjacent tissue. (B) Correlation between *SOX17* and *TNS4* mRNA expression in TCGA cohorts. For each cohort from TCGA, we converted the "scaled estimate" in the "illuminaiseq_rnaseqv2_unc_edu_Level_3_RSEM_genes" file into TPM by multiplying by 10^6 .

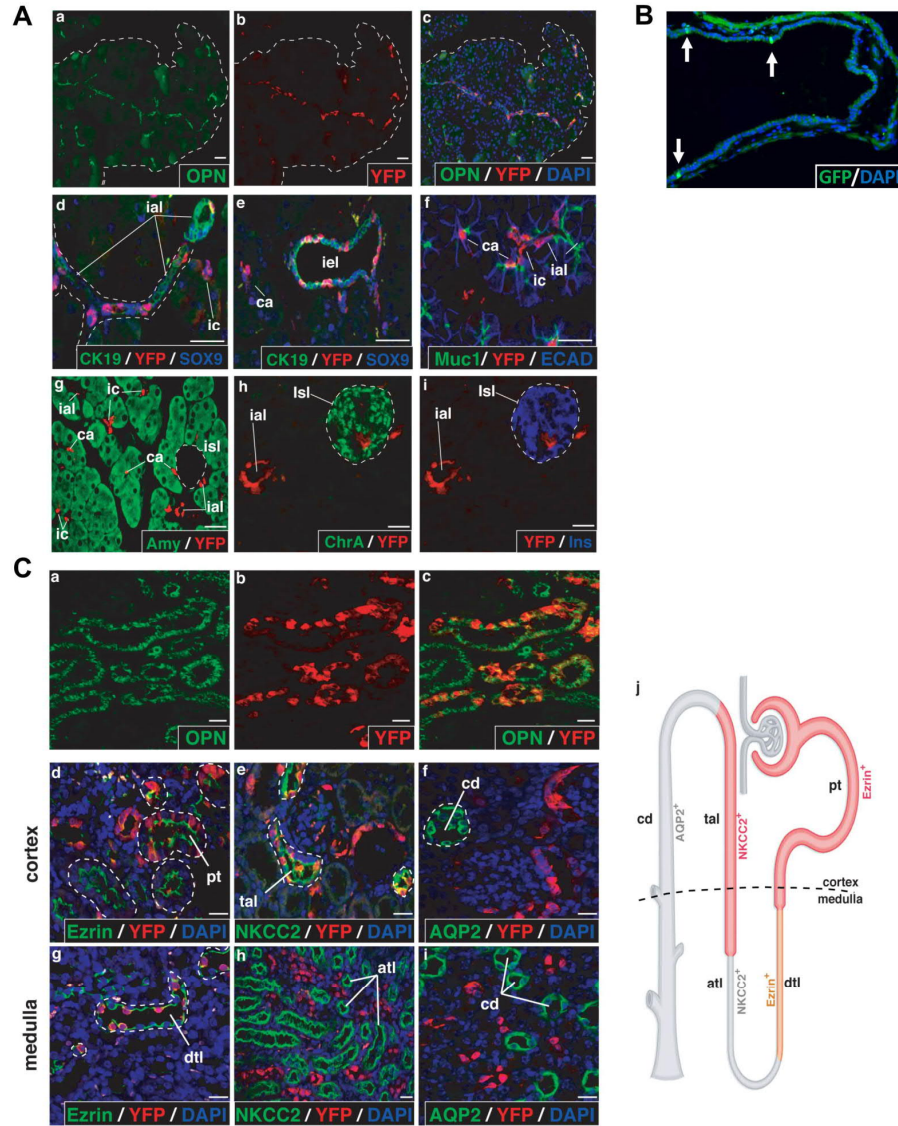


Fig. S5. OPN-iCreERT² activity 6-8 week-old OPN-iCreERT²;Rosa26R^{eYFP} mice after tamoxifen injection. (A) In pancreas eYFP expression is induced only in OPN-expressing cells (A, a-c). eYFP is detected in centroacinar cells (ca), and in intercalated (ic), intralobular (ial) and interlobular (iel) ducts (A, d-f); eYFP is co-expressed with Muc-1 and SOX9; ECAD, E-cadherin. Amylase (Amy)-positive cells are eYFP-negative (A,g). YFP-positive cells within islets are chromogranin A (ChrA)- and insulin (Ins)-negative and do not belong to the endocrine lineage (A,h-i). (B) In the gallbladder very few epithelial cells (arrows) express eYFP. (C) In kidney, induction of eYFP is restricted to OPN-positive cells (C,a-c). eYFP is induced in cortical segments expressing ezrin or Na-K-Cl cotransporter 2 (NKCC2), i.e. in the proximal tubules (pt) and thick ascending limbs (tal), respectively, but not in Aquaporin-2 (AQP2)-positive collecting ducts (cd) (C,d-f). In the medulla, eYFP is induced in ezrin-positive descending thin limbs (dtl), but not in NKCC2-positive ascending thin limb (atl) or AQP2-positive collecting ducts (cd) (C,g-i). Tubular segments in the kidney; eYFP is detected in the red- and orange-stained segments (C,j). Scale bars: 20 μ m.

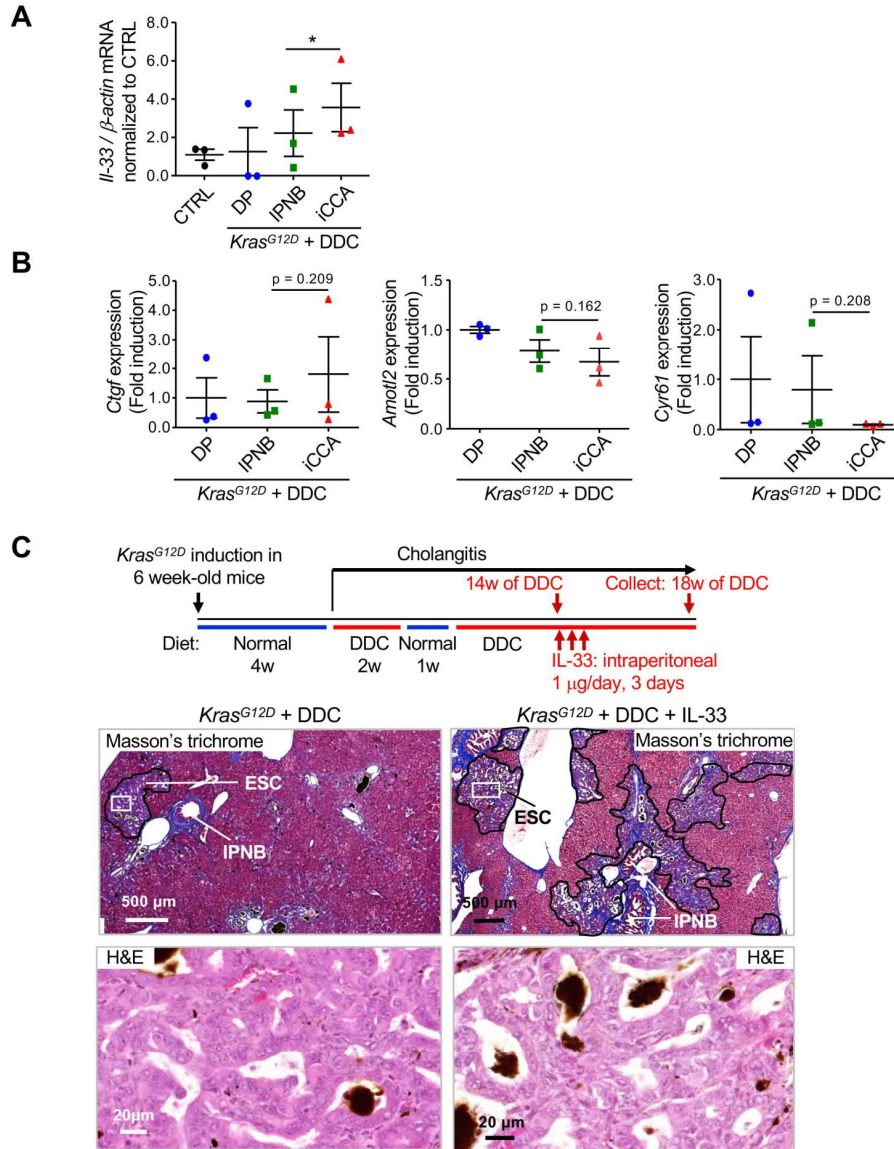


FIG. S6. Role of IL-33 in tumor progression. (A) Expression of IL-33 mRNA measured by RT-qPCR in ductular proliferations (DP), intraductal papillary neoplasms of the bile ducts (IPNB) and intrahepatic cholangiocarcinoma (iCCA) microdissected from Opn-Cre^{ER}/LSL-Kras^{G12D} mice treated with tamoxifen to induce Kras^{G12D} and fed a DDC diet for 25 weeks. Control cholangiocytes (CTRL) were FACS-purified from tamoxifen-treated Opn-Cre^{ER}/Rosa26R^{YFP} mice. Data are means of fold inductions $\pm$ SEM. * $p < 0.05$. (B) Expression of Hippo-Yap signaling markers *Ctcf*, *Amotl2* and *Cyr61*. Data are normalized counts (means $\pm$ SEM) from RNAseq analysis of RNA microdissected from DP, IPNB, iCCA from Opn-Cre^{ER}/LSL-Kras^{G12D} mice treated with tamoxifen to induce Kras^{G12D} and fed a DDC diet for 25 weeks. (C) Histology of Opn-Cre^{ER}/LSL-Kras^{G12D} mice treated with tamoxifen, fed a DDC diet and injected with IL-33 as indicated. Bottom panels show high magnifications of white boxed regions in upper panels, on adjacent sections stained with hematoxylin and eosin. A 23-fold increase in the area of early stage cancer (ESC) lesions was monitored ($n = 3$, 507 areas investigated) as compared to tamoxifen- and DDC-treated Opn-Cre^{ER}/LSL-Kras^{G12D} mice which, in parallel, had not received IL-33 ($n = 3$ mice; 513 areas investigated). The lesions did not show histological progression toward further stages of malignancy.

III.2 Gallbladder cancer

III.2.1 Mouse models of gallbladder carcinogenesis

Since most efforts in developing animal models of biliary tract cancer have been focused on iCCA, we are currently confronted with a lack of animal models of GBC that faithfully recapitulate human histology. Earlier publications report the development of GBC in hamsters or mice treated with toxicants, in combination or not with the introduction of pellets in the gallbladder to mimic gallstone disease (74-77). Unfortunately, the nature of the lesions produced in these models is difficult to assess. Kiguchi et al. described the occurrence of GBC in a transgenic mouse model with constitutive overexpression of ERBB2 in the gallbladder epithelium driven by the bovine Keratine 5 promoter (78). Our analysis of the published data concludes that the lesions described as GBC consist rather in exophytic papillary proliferations towards the lumen of the gallbladder with only a few glands suspect for micro-invasion. Moreover, this proliferation starts 2 weeks after birth because the transgene is already expressed in prenatal life, which is not optimal to mimic a cancer normally occurring in adults. Gabbi et al. generated another mouse model of GBC with inactivation of the oxysterol receptor liver X-b (LXR-b ^{-/-}), a nuclear receptor involved in lipid homeostasis, glucose metabolism, inflammation and proliferation (79). They report the development of preneoplastic and malignant lesions in the gallbladder of female mice. The gallbladder of male LXR-b ^{-/-} mice was indistinguishable from wild type mice, which, in line with epidemiology of GBC, would suggest the involvement of estrogens in gallbladder carcinogenesis. This was further claimed to be supported by the observation that ovariectomy in female LXRb ^{-/-} mice prevented the appearance of the phenotype. The presence of the phenotype in female mice recapitulates the preponderance of GBC in women. Therefore, LXR-b is an interesting target to consider in the pathogenesis of GBC. However, our analysis of the published data does again not conclude that the lesions convincingly correspond to BilIN, adenoma or GBC. Instead, the animals display obvious signs of chronic cholecystitis and the histological images do not support the existence of adenoma or carcinoma in situ which I believe is essential for a model aimed to induce GBC. .

In that context, we aimed to develop a model of gallbladder cancer recapitulating human morphology to study cancer development from precursor

lesions in vivo and in situ by postnatal induction of mutations described in human GBC.

III.2.1.1 Mouse Cre^{ER} models for inducible expression of tumor-promoting genes in gallbladder epithelium

The Opn gene is expressed in the gallbladder, suggesting that tamoxifen-treated OpnCre^{ER} LSL-Kras^{G12D} mice used for studying iCCA may express KRAS^{G12D} in the gallbladder epithelium and potentially develop proliferative lesions. Except for a single mouse which had been fed a DDC diet for 31 weeks and which presented non-invasive papillary proliferation (Figure 1A), we did not observe any macroscopic lesion in the gallbladder of other OpnCre^{ER} LSL-Kras^{G12D} mice, even fed a DDC diet. This may result from the surprisingly low efficiency of OpnCre^{ER}-mediated gene recombination induced in gallbladder. Indeed, Figure 1B illustrates that, following tamoxifen administration, only rare cells express eYFP in the gallbladder epithelium of OpnCre^{ER} LSL-Kras^{G12D} LSL-Rosa^{YFP} mice fed a DDC diet for 12 weeks (Figure 1B). We therefore assumed that the proliferative lesion illustrated in Figure 1A results more likely from a reactive process towards DDC plugs present in the lumen of the gallbladder, rather than from expression of KRAS^{G12D}.

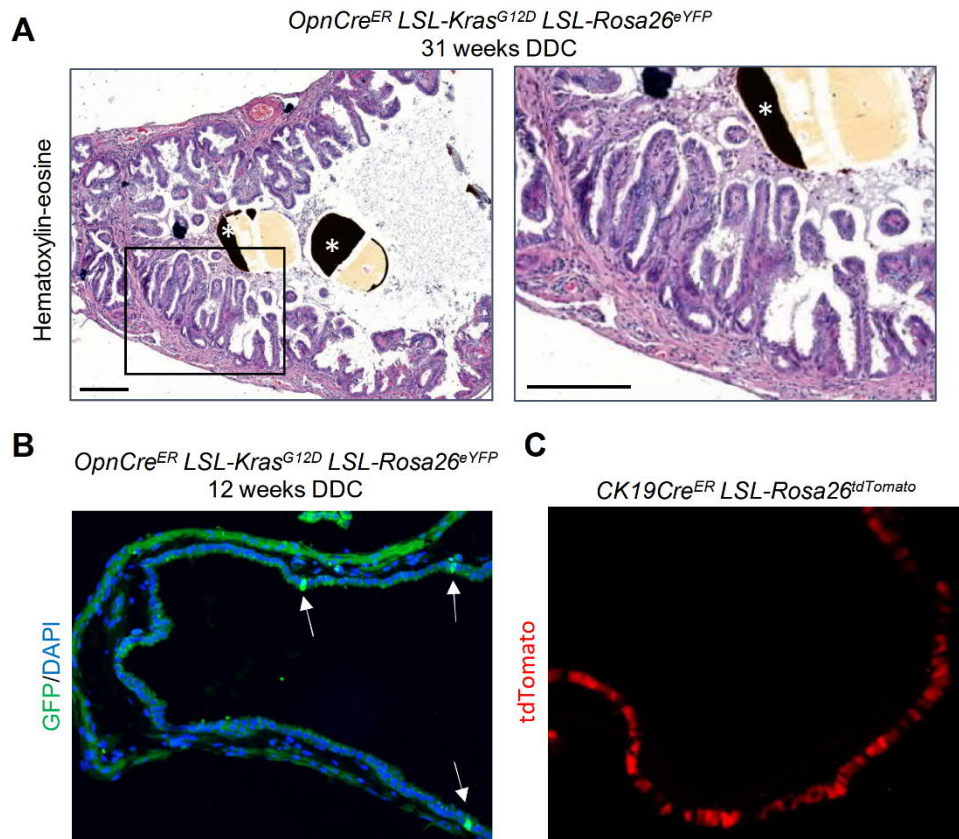


Figure 1. Activity of *OpnCre^{ER}* and *CK19Cre^{ER}* in mouse gallbladders. (A) Non invasive papillary proliferation in the gallbladder of an *OpnCre^{ER} LSL-Kras^{G12D} LSL-Rosa26^{eYFP}* mouse fed a DDC diet for 31 weeks. The box in the left panel is magnified in the right panel. *, DDC plugs in the lumen of the gallbladder. Size bar is 200 μ m. (B) Gallbladder from an *OpnCre^{ER} LSL-Kras^{G12D} LSL-Rosa26^{eYFP}* mouse fed a DDC diet for 12 weeks showing 3 YFP-positive cholangiocytes (arrows). (C) Gallbladder from a *CK19Cre^{ER} LSL-Rosa26^{tdTomato}* mouse showing expression of the tdTomato protein in the majority of the cholangiocytes. Direct fluorescence of tdTomato is detected.

The low efficiency of gene recombination obtained with *OpnCre^{ER}* prompted us to consider the use of *CK19Cre^{ER}*. *CK19Cre^{ER}* had indeed been used to induce eCCA (23), and our analysis of *CK19Cre^{ER} Rosa^{tdTomato}* mice indeed

demonstrated that this Cre^{ER} line is much more suitable for investigating gene function in the gallbladder epithelium (Figure 1C).

III.2.1.2 Selection of potential gallbladder tumor-promoting genes and generation of transgenic mice

KRAS^{G12D} mutations are reported in gallbladder cancer (80). While the lack of gallbladder tumor development in OpnCre^{ER} LSL-Kras^{G12D} LSL-Rosa^{YFP} mice likely results from low efficiency of Kras^{G12D} induction, we also considered that expression of this mutant might not be sufficient to induce tumorigenesis. Consequently, we decided to induce Kras^{G12D} in combination with other mutations known to occur in human GBC. Several reasons prompted us to select TP53. Indeed, TP53 mutations are the most frequent in GBC (80) and are early events in gallstone-induced gallbladder carcinogenesis (66, 81). The sequencing data of Li et al. show a majority of samples with TP53 missense mutations in GBC (80). In human somatic cancers in general, TP53 missense mutations account for 72.7% of all TP53 mutations. TP53^{R175H} is the most frequent mutational hotspot in human somatic cancer (82) and Li et al. found this missense mutation in GBC. TP53^{R175H} is a DNA binding domain mutant inducing a conformational change and a decrease of DNA binding capacity that predisposes to cancer (83).

Because of the efficiency of the CK19Cre^{ER} system in gallbladder epithelium and of the reported importance of the TP53^{R175H} mutation in human gallbladder carcinogenesis, we decided to generate CK19Cre^{ER} LSL-p53^{R172H/+} mice and to mate them with LSL-Kras^{G12D} LSL-Rosa26^{eYFP} mice to obtain animals combining expression of eYFP, KRAS^{G12D} and TP53^{R172H} in the gallbladder epithelium (Figure 2A).

ERBB3 missense mutations are the second most common mutations in GBC (80). The most frequent mutations consist in ERBB3^{V104M} or ERBB3^{V104L}, which modify the extracellular domain of the receptor. The ERBB signaling pathway is identified as the most extensively mutated in GBC (80). Interestingly, ERBB3^{V104M} is a mutational hotspot in multiple human cancers such as colorectal and gastric cancers. It has displayed oncogenic potential in mice. Immortalized mouse colonic epithelial cells expressing the mutant ERBB3^{V104M} present an anchorage-independent growth and an accelerated tumor growth in xenograft mouse models,

when co-expressed with ERBB2 (84). ERBB3^{V104L} induces significant increase in cell proliferation of GBC cell lines expressing ERBB2 (80). The oncogenic effects of the ERBB3^{V104M} and ERBB3^{V104L} mutants were observed in the absence of the ERBB3 ligands (the neuregulins, mainly NRG1) but required ERBB2, consistent with the need for ERBB3 to partner with an effective tyrosine kinase domain to acquire signaling abilities (ERBB3 tyrosine kinase domain is deficient). Given the frequency of the ERBB3 mutations at aminoacid 104 in GBC, we generated a new mouse line expressing inducible ERBB3^{V104M} mutation (Figure 2B). This line was generated at the UCLouvain transgenic core facility (Dr. Younes Achouri). These mice were mated with CK19Cre^{ER} LSL-p53^{R172H/+} to combine expression of TP53 and ERBB3 mutants (Figure 2C).

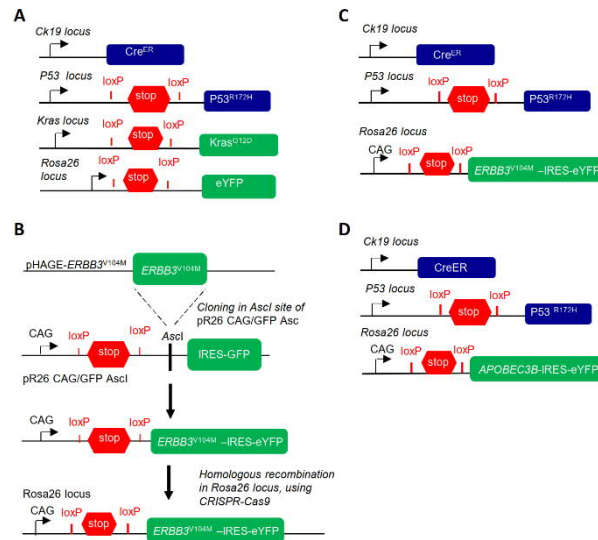


Figure 2. Transgenic mouse lines. (A) CK19Cre^{ER} LSL-p53^{R172H} LSL-Kras^{G12D} LSL-Rosa26^{eYFP}. (B) Strategy to generate a mouse line expressing inducible ERBB3^{V104M}. Human ERBB3^{V104M} cDNA was cloned from pHAGE- ERBB3^{V104M} (Addgene # 116363) in pR26 CAG/GFP AscI (Addgene # 74285) and introduced with the CAG promoter, IRES-GFP and a loxP-flanked stop cassette in the Rosa26 locus by homologous recombination using CRISPR/Cas9. (C) CK19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{ERBB3(V104M)-IRES-eYFP}. (D) CK19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP}

Finally, the APOBEC3B-mediated mutational signature is found in biliary tract cancers (14) and contributes more to the mutational load in GBC than in eCCA and iCCA. The level of expression of APOBEC3B also positively correlates with the proportion of mutations attributed to APOBEC3B in biliary tract cancers (14, 80) suggesting the importance of this enzyme in gallbladder carcinogenesis. APOBEC3B displays cytidine deaminase activity, inducing DNA mutations in the specific nucleotide motif 5' - TC^A/G - 3', causing cytosine to uracil transitions. APOBEC3B is the only APOBEC isoform that is constitutively located in the nucleus and cytoplasm, suggesting a possible role in DNA damaging or mutation leading to cancer (85). Because of the oncogenic potential of APOBEC3B, we aimed to overexpress the human APOBEC3B gene in combination with expression of TP53^{R172H}. LSL-Rosa26^{APOBEC3B-IRES-eYFP} mice (kind gift from Nicolas Gillet, UNamur) were mated with CK19Cre^{ER} LSL-p53^{R172H/+} mice (Figure 2D).

These three mouse models were created in a mixed background of CD1 and C57BL/6 and received intraperitoneal injections of Tamoxifen 7 weeks after birth (67.5mg of Tamoxifen in 3 injections) to induce the expression of the transgenes.

III.2.1.3 Gallbladder phenotype of transgenic mice

Ck19Cre^{ER} LSL-p53^{R172H} LSL-Kras^{G12D} mice suffered from multiple cancers, especially spindle and pleiomorphic cell tumors possibly compatible with sarcomas and lung cancers, imposing early euthanasia (Figure 3A-B). The gallbladders were normal 3.5 months after tamoxifen injection (Figure 3C) and we decided not to pursue the analysis of this mouse model. The finding of sarcomas in a Ck19Cre^{ER} system was unexpected but could be explained by the fact that the Ck19 is also expressed in striated muscle fibers (86).

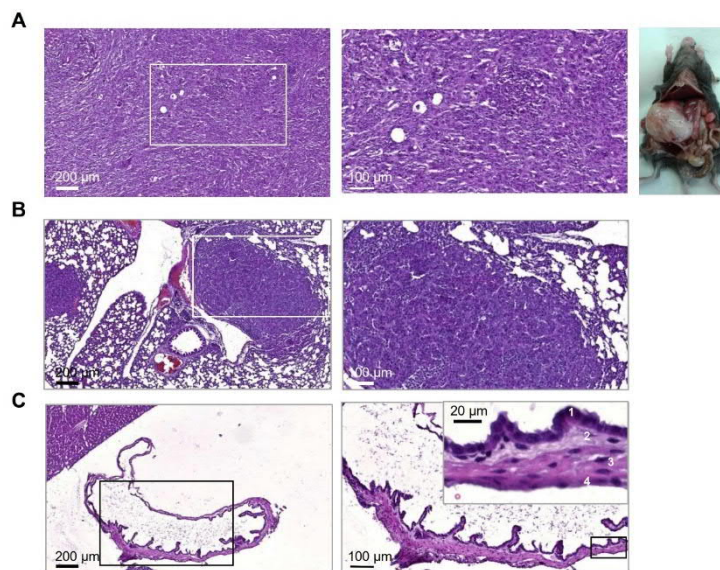


Figure 3. Phenotypic analysis of Ck19Cre^{ER} LSL-P53^{R172H} LSL-Kras^{G12D} mice. Seven-week old mice were injected with tamoxifen and analyzed 3.5 months after injection (n=2). (A) Retroperitoneal tumor composed of spindle and pleiomorphic cells possibly compatible with a sarcoma. (B) Multiple lung tumors compatible with lung cancer were detected in the same mouse at the same time point. (C) The gallbladder of the mouse was histologically normal. 1. epithelium: 2.

No histological lesion was detected in the gallbladder of Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{ERBB3(V104M)}-IRES-eYFP mice even 9 months after tamoxifen injection (Figure 4). This raised the question of the expression of the mutant ERBB3^{V104M} protein in the Ck19Cre^{ER} Rosa26^{ERBB3(V104M)}-IRES-eYFP model. Because immunohistochemical detection of the ERBB3^{V104M} protein was not possible using available antibodies, we used real time RT-PCR to verify expression of ERBB3^{V104M}. Gallbladder and cystic duct of Ck19Cre^{ER} Rosa26^{ERBB3(V104M)}-IRES-eYFP mice were collected 5 days after tamoxifen injection and total RNA was extracted (n=5). A single mouse expressed detectable ERBB3^{V104M} mRNA (Ct= 23.15), while no ERBB3^{V104M} mRNA was detected in the other 4 mice. RT-PCR analysis of eYFP mRNA revealed variable expression in Ck19Cre^{ER} Rosa26^{ERBB3(V104M)}-IRES-eYFP mice gallbladders. This was unexpected but the degree of variability unfortunately led us to conclude that the mouse model was inappropriate to investigate the role of ERBB3^{V104M} in GBC tumorigenesis.

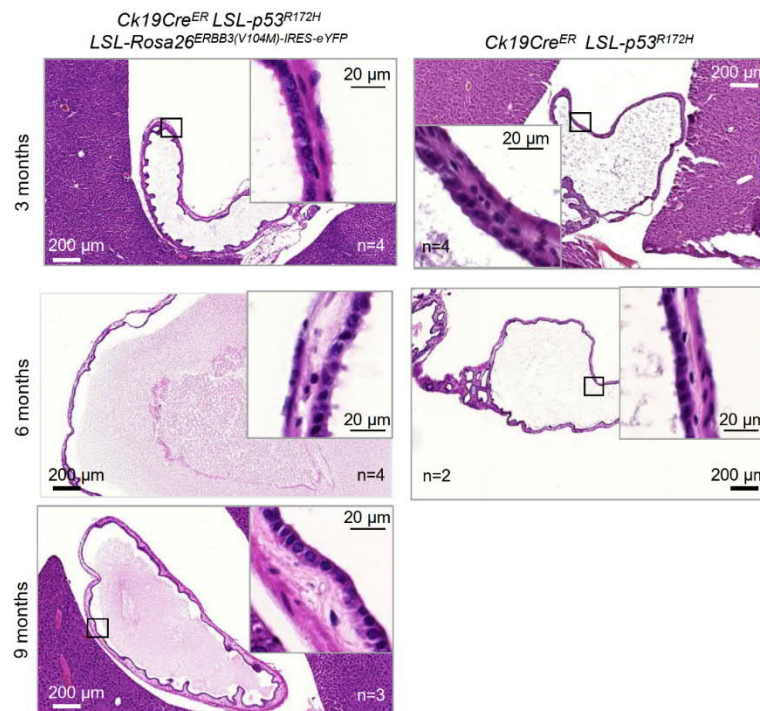


Figure 4. Ck19Cre^{ER} LSL-P53R172H and Ck19Cre^{ER} LSL-P53R172H LSL-Rosa26ERBB3(V104M)-IRES-eYFP mice do not present with detectable gallbladder phenotype. The mice were analyzed at the indicated time points after tamoxifen injection

We next investigated the role of APOBEC3B overexpression in combination with TP53 mutation. Here again, no phenotype was detected in the gallbladder of Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} or Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mice (Figure 5). The transgenic APOBEC3B protein is detected by immunohistochemistry in gallbladders (Rabbit anti-Apobec3B antibody, ref. 5210-87-13). However, the number of cells expressing APOBEC3B in the gallbladder epithelium varied considerably from one mouse to the other, in both mouse lines (Figure 6A-B).

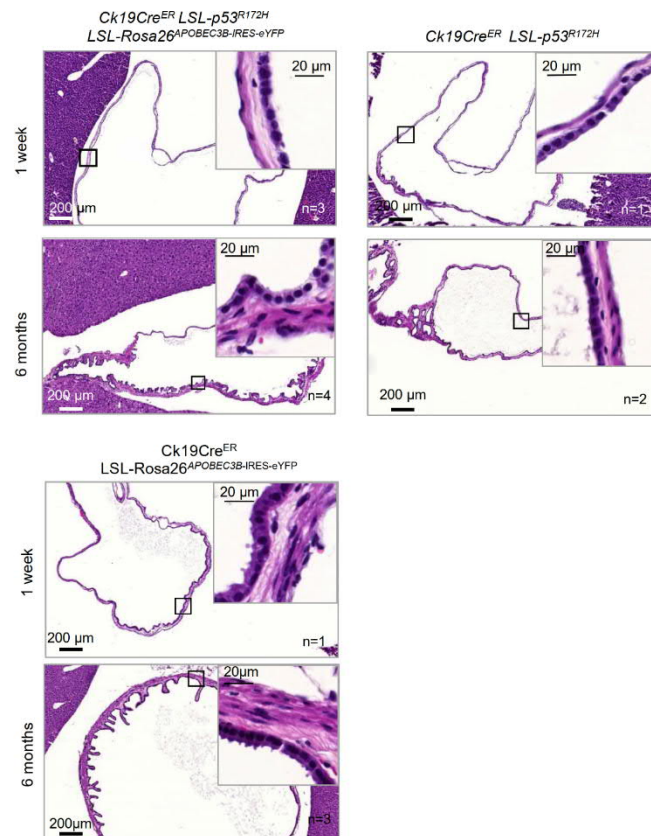


Figure 5. Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP}, Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} and Ck19Cre^{ER} LSL-p53^{R172H} mice do not present with detectable gallbladder phenotype. The mice were analyzed at the indicated time points after tamoxifen injection. Epithelial folds reflect the lack of gallbladder filling with bile.

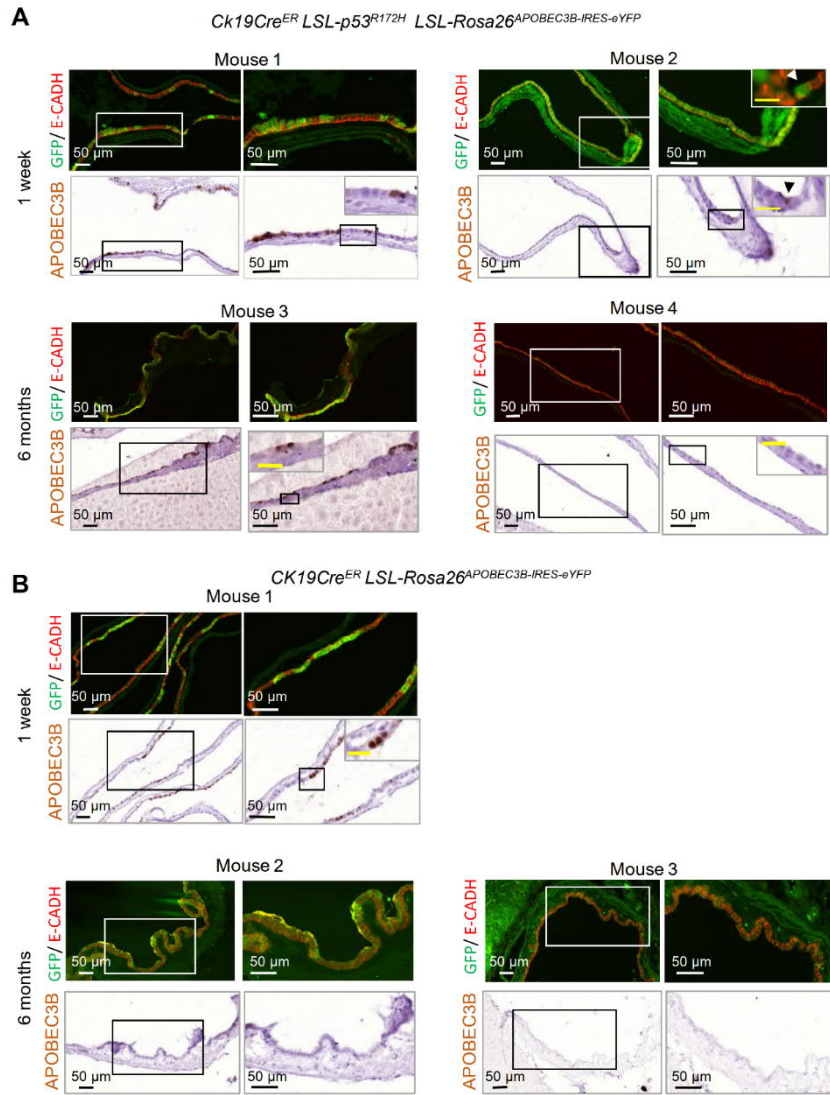


Figure 6. Variable expression of APOBEC3B in murine gallbladders from *Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP}* mice and *Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP}* mice. (A) Patches of cells expressing APOBEC3B, colocalized with GFP, are detected 1 week after tamoxifen injection in one of the two *Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP}* mouse gallbladders (mouse 1). Mouse 2 only shows one APOBEC3B⁺/GFP⁺ cell (arrowhead). Six months after tamoxifen injection, the expression of APOBEC3B and GFP is detected in the gallbladder of mouse 3, not in mouse 4. (B) The expression of APOBEC3B and GFP is detected 1 week after tamoxifen injection in one *Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP}* mouse (mouse 1) but not 6 months later (mice 2 and 3). Yellow size bar is 20 μ m.

Several hypotheses could explain this variable APOBEC3B expression. First, Ck19Cre^{ER} may recombine APOBEC3B and eYFP genes with variable efficiency. This would also explain the variable expression of the transgenic ERBB3^{V104M} mRNA in Ck19Cre^{ER} Rosa26^{ERBB3(V104M)-IRES-eYFP} mice. Second, the expression of the mutant TP53^{R172H} protein may impact the expression of APOBEC3B because one Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mouse still expresses the recombinant APOBEC3B protein 6 months after tamoxifen injection while Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mice expressing the wild type TP53 do not express APOBEC3B 6 months after Tamoxifen injection (Figure 6). APOBEC3B is known to induce cell death and there is a positive correlation between the levels of expression of APOBEC3B and TP53 inactivation in breast cancer, suggesting that TP53 inactivation is required to allow APOBEC3B expressing cells to bypass cell death (87). This hypothesis could explain the variable APOBEC3B expression between both genotypes (Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} versus Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP}) 6 months after tamoxifen injection. However, it does not explain the variable expression observed within the same group of mice. Third, apical extrusion is a mechanism of epithelial defense against cancer by which normal epithelial cells actively eliminate newly transformed (mutated) cells. Apically extruded cells present a nucleus displaced towards the lumen and seem to be pushed out of the epithelium (88). We detected scarce GFP-positive cells whose nucleus is displaced at the apical pole of the cell towards the lumen of the gallbladder (Figure 7).

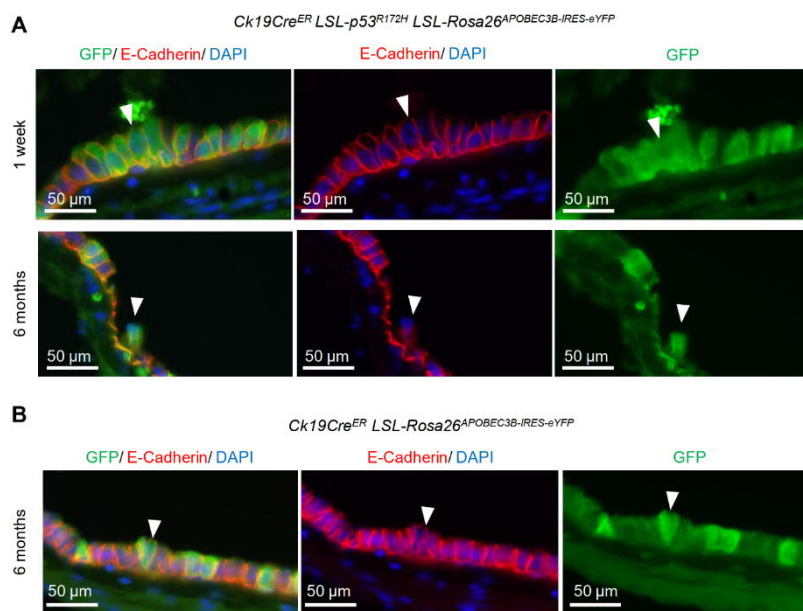


Figure 7. Potentially extruded cholangiocytes from Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} and Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} murine gallbladders. (A) GFP-positive cholangiocytes (arrowheads) showing a nucleus displaced towards the lumen of the gallbladder in Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mice. (B) GFP- positive cholangiocyte (arrowhead) displaying a morphological aspect compatible with apical extrusion in a Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mouse gallbladder

These potentially extruded cells are observed in Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} and Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mice gallbladders at different time points. Apical extrusion could thus partly contribute to the variable detection of APOBEC3B. Still, given the limited number of cells displaying criteria of- cellular extrusion, this mechanisms is unlikely to be the main cause of variable APOBEC3B detection.

In conclusion, Ck19Cre^{ER} LSL-p53^{R172H} LSL-Kras^{G12D} mice were not considered appropriate to study gallbladder carcinogenesis because the mice suffer from multiple other cancers imposing early euthanasia, i.e. before any potential lesion develops in the gallbladder. Further, our investigation of mice expressing mutant ERBB3^{V104M} or overexpressing APOBEC3B was confronted with highly variable expression of the corresponding mRNA or protein. Since we were attempting to develop a mouse model which develops GBC within a reasonable period of time and with faithful recapitulation of human GBC histology, we had to conclude that neither of the studied models fitted with our expectations.

III.2.2 Spatial transcriptomics of human gallbladder cancer and biliary intraepithelial neoplasia

Spatial transcriptomic profiling of gallbladder adenocarcinoma development reveals limited intra-patient variability within precursor and cancer lesions and uncovers patient-specific histogenic paths of tumour progression

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Data availability: The raw dataset generated from GeoMx DSP analysis during the current study is available in Supplementary Material.

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Conflict of interest: The authors declare no competing interests.

Ethics approval: The study on human samples was conducted in compliance with the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Comité d'Ethique Hospitalo-Facultaire (UCLouvain and Cliniques Universitaires Saint-Luc) with numbers 2018/06Jul/281 and 2021/26OCT/444. Mice received humane care and the research protocol was approved by the Animal Welfare Committee of the Université Catholique de Louvain with number 2022/UCL/MD/17.

Patient consent: In accordance with article 8 of the internal rules of the Cliniques universitaires Saint-Luc, the need for informed consent was waived to the present retrospective study. The study is based solely on the analysis of residual human body material and on the collection of data existing in the medical files of patients who have not expressed their opposition to the use of their medical file for scientific research purposes. An informed consent exemption request was thus presented to the Ethics Committee, which was accepted.

Running title: Spatial transcriptomics of gallbladder cancer

Abstract

Genomic and epigenomic studies identified distinct tumourigenic paths leading to gallbladder adenocarcinoma, a subset of cancers originating from biliary intraepithelial neoplasia (BillIN) precursor lesions, whereas others form independently from BillINs. Spatial transcriptomic data confirming these conclusions are missing. Moreover, multiple areas with precursor or adenocarcinoma lesions can be detected within the same pathological sample. Yet, knowledge about intra-patient variability of such lesions is lacking. To characterise the spatial transcriptomics of gallbladder cancer tumourigenesis, with emphasis on intra-patient variability and tumour progression mechanisms, we selected two patients with distinct cancer etiology and whose samples simultaneously displayed multiple areas of normal epithelium, BillINs and adenocarcinoma. Using GeoMx digital spatial profiling, we characterised the whole transcriptome of a high number of regions of interest (ROIs) per sample in the two patients (24 and 32 ROIs respectively), with each ROI covering approximately 200 cells of normal epithelium, low-grade BillIN, high-grade BillIN or adenocarcinoma. We found that despite that they were scattered throughout the tissue sample, each type of lesion displayed limited intra-patient transcriptomic variability. Our data further suggest that adenocarcinoma can derive from high-grade or low-grade BillIN, with co-existing high-grade BillIN evolving via a distinct process in the latter case. The two patients displayed distinct sequences of signalling pathway activation during tumour progression, thereby supporting the existence of patient-specific tumourigenic mechanisms. SEMAPHORIN 4A (*SEMA4A*) expression was repressed in both patients. Using organoids and cell line-derived tumours, we provide evidence that repression of *SEMA4A* enhances cell migration and survival, and perturbs polarisation. In conclusion, our results support that gallbladder adenocarcinoma can be promoted by repression of *SEMA4A* and develops according to patient-specific processes with limited intra-patient variability of precursor and cancer lesions. They highlight the need to gain gene expression data in addition to histological information to avoid underestimating the risk of low-grade preneoplastic lesions.

INTRODUCTION

Gallbladder cancer accounts for less than 2% of cancer-related deaths and is often fortuitously diagnosed in gallbladder samples following cholecystectomy. The prognosis of the disease remains poor because patients often present at an advanced stage with unresectable tumour. Late diagnosis results from the lack of specific symptoms and of screening strategies, as well as from limited knowledge of the mechanisms driving tumour progression [1, 2]. Several studies investigated the pathology, genomics and epigenomics of tumour progression. However, spatial transcriptomic data corroborating their conclusions are missing. Moreover, multiple areas with precursor or adenocarcinoma lesions may be detected within the same pathological sample. In that context, a gap of knowledge remains in our characterisation of intra-patient variability of such lesions.

Adenocarcinomas account for >90% of gallbladder cancers and are considered to develop according to a metaplasia-dysplasia-adenocarcinoma histogenic sequence, in which the dysplastic stage consists of low-grade and high-grade biliary intraepithelial neoplasia (BillN) [3-6]. BillNs consist of microscopic, flat or micropapillary lesions whose grade depends on the highest degree of cytological and architectural atypia. Low-grade BillNs display moderate cytoarchitectural atypia with pseudostratification of the nuclei, increased nucleo-cytoplasmic ratio and hyperchromasia. High-grade BillNs, formerly called carcinomas *in situ*, are defined by loss of nuclear polarity, marked cytological atypia and complex architectural patterns such as micropapillae [7-9].

Genomic alterations are already found at the BillN stage. *KRAS* mutations were found in BillNs [10] and a recent exome sequencing study uncovered *CTNNB1*, *TP53*, *ARID2* and *ERBB3* as the most frequently mutated genes in low-grade and high-grade BillNs [11]. When the disease evolves to invasive adenocarcinoma, alterations accumulate. At that stage the most frequent mutations affect *KRAS*, *CTNNB1*, *TP53*, *PI3KCA*, *ERBB2*, *CDKN2A* and *CDKN2B* [12-20], indicating that a fraction of the mutations found at the cancer stage can be detected in BillN

lesions. At the epigenome level, cancer lesions were split in subtypes with distinct hypermethylation:hypomethylation ratios; progressive and cumulative changes in promoter methylation were detected during progression from cholecystitis to cancer [20, 21]. Increased hypermethylation was observed in adenocarcinomas as compared with BillNs. These epigenomic changes impacted Wnt/ β -catenin signalling, Hedgehog signalling, tumour suppression and cell-microenvironment interactions [22-24]. Further, since gallstone-induced chronic inflammation drives gallbladder carcinogenesis [25], several authors compared the transcriptome of normal gallbladder tissue, gallbladder cancer, and gallbladder tissue exposed for varying lengths of time to gallstones, and identified molecular signatures associated with disease progression [26, 27]. Finally, in line with the genomic and epigenomic studies, single gene analyses revealed aberrant expression levels of P53, P21, cyclin D1, EZH2, SMAD4 and CDKN2A at the BillN stage [9], as well as the ability of a combined activation of KRAS and canonical Wnt/ β -catenin or Notch signalling to induce gallbladder BillNs with malignant potential [28, 29]. Spatial transcriptomic data investigating BillN to adenocarcinoma progression are still lacking.

Considering the genomics of tumour progression, Lin and coworkers provided evidence for patient-specific tumourigenic processes [11]. Their results indicated that precursor and cancer lesions within the same patient bear similar mutations, whereas the mutational signatures significantly vary between patients. Phylogenetic analysis of single nucleotide variants in lesions generated revealed that gallbladder cancer developed either BillN-dependently or BillN-independently [11].

To address the spatial transcriptomics of gallbladder tumour progression, while emphasising both the intra-patient variability and the tumour progression mechanisms, we selected samples from two patients displaying simultaneously several areas of gallbladder BillNs and adenocarcinoma, and collected an extensive spatial transcriptomic data set of each type of lesion per patient. The two patients were selected because of their differing cancer etiology,

offering the possibility to address intra-patient variability and tumour progression in distinct contexts. Our results show that each type of lesion displayed limited variability within the same patient, but significantly differed among patients. This revealed that the two patients have distinct tumourigenic processes, thereby corroborating earlier conclusions at the transcriptomic level. Our molecular investigations using organoids and cell line-derived tumours also provide evidence that reduced SEMAPHORIN 4A (SEMA4A) expression, which was observed in the two patients, promotes tumour progression.

METHODS

Ethics approval

Human samples were studied in compliance with the guidelines of the 2013 Declaration of Helsinki and was approved by the Comité d'Ethique Hospitalo-Facultaire (Université Catholique de Louvain and Cliniques Universitaires Saint-Luc; 2018/06Jul/281 and 2021/26OCT/444). The study is based solely on the analysis of residual human body material and on the collection of data existing in the medical files of patients who have not expressed their opposition to the use of their medical file for research purposes. In accordance with article 8 of the internal rules of the Cliniques universitaires Saint-Luc, the need for informed consent was waived to the present retrospective study. The research protocol using mice was approved by the Animal Welfare Committee of the Université Catholique de Louvain (2022/UCL/MD/17).

Spatial transcriptomics

Spatial profiling was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections using GeoMx (NanoString Technologies, Seattle, WA, USA) [30] which was implemented by NanoString. The GeoMx Whole Transcriptome Atlas assay probe cocktail containing 18,677 probes was tested. ROIs subjected to spatial transcriptomic profiling encompassed epithelial areas of approximately 200 cells. The 24 ROIs of Patient #1 were all located on the same tissue section. For Patient #2, the ROIs were partitioned over two sections, namely 8 ROIs

covering normal epithelium on one section, and 24 ROIs covering lesional tissue on a second section ([Table 1](#)).

Histology, immunofluorescence and immunohistochemistry

Haematoxylin/Eosin (H&E) staining, immunofluorescence and immunohistochemistry were performed on sections of FFPE tissues or organoids. Primary and secondary antibodies are described in [supplementary material, Table S1](#).

Gallbladder organoid culture

Human non-tumoral gallbladder tissues were obtained from patients who underwent cholecystectomy at the Cliniques Universitaires Saint-Luc, Brussels, using the method of Rimland and coworkers [31]. The karyotype of the selected organoid line was normal and whole exome sequencing detected an ERBB3^{R675G} missense mutation at an allelic fraction of 0.021. To analyse the impact of blocking SEMA4A in gallbladder organoids, the latter were split and plated. After 24 h, SEMA4A antibody (IgG-SEMA4A, #14-1002-82 eBioscience/Thermo Fisher scientific, Brussels, Belgium) was added into the medium (10 µg/ml) and organoids were grown for 3 days.

Clonogenic and transwell assays

Colony forming capacity was determined by seeding 200 cells per well of a 6-well plate. Colony formation was allowed to occur over 10 days under 50 ng/ml treatment of recombinant human protein SEMA4A (rhSEMA4a; Abcam #ab182683, Amsterdam, The Netherlands) or 10 µg/ml of IgG-SEMA4A, with the medium replenished every 3 days. Cell migration was evaluated by using 8.0 µm pore size transwell inserts (Corning/Merck, Hoeilaart, Belgium) in 24-well plate wells. Prior to migration assessment, cells were pre-treated for 48 h with 50 ng/ml rhSEMA4a or 10 µg/ml of IgG-SEMA4A. Next, cells were seeded into the upper chamber transwell insert

(6×10^4 cells) in 200 μ l serum-free medium while 750 μ l medium with 10% foetal bovine serum was added to the lower chambers. In the case of cells treated with rhSEM4A or IgG-SEMA4A, both chambers contained rhSEM4A (50 ng/ml) or IgG-SEMA4A (10 μ g/ml), respectively. The area covered by migrated cells was analysed using ImageJ software 1.50 (NIH, Bethesda, USA).

***In vivo* assessment of SEMAPHORIN 4A function**

10^6 EGI-1 cells were injected subcutaneously into the flanks of NSG mice. After 4 weeks, the animals received intraperitoneal injections of PBS, IgG-SEMA4A (5 mg/kg), or rhSEM4A (500 μ g/kg) every other day, receiving a total of 4 doses of treatment. Tumour volume (V) was measured every 48 h. Relative tumour volume was defined by normalising to the initial tumour volume at the start of the treatment (V_0).

Mice

NOD-SCID-gamma (NSG) mice were housed under a 12 h light/dark cycle in individually ventilated cages supplied with RM3 chow (#801700, Tecnilab, Someren, Netherlands), acidified water, and polyvinyl chloride play tunnels. Animals were monitored daily and received humane care according to the Directive 2010/63/EU of the European Parliament, and to the Belgian law of May 29, 2013 updated on September 7, 2017 by the Brussels Region.

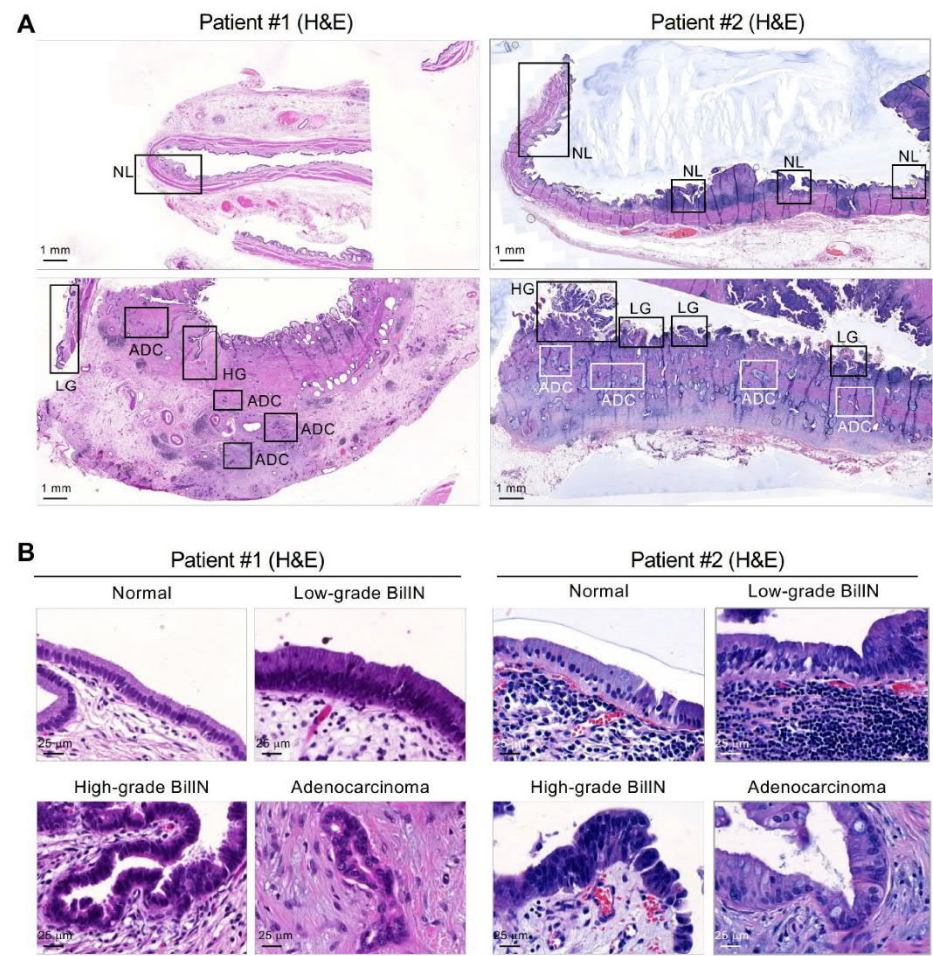
Further details are in Supplementary Methods.

RESULTS

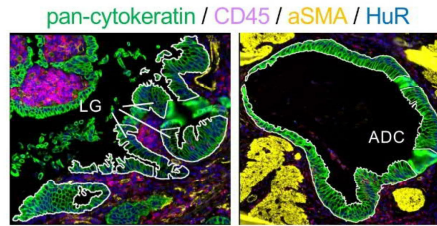
Selection of normal epithelium, BillIN and adenocarcinoma in samples of human gallbladder

Our goal is to characterise the spatial transcriptome of gallbladder lesions during progression from normal epithelium to adenocarcinoma, while focusing on intra-patient transcriptomic variability and tumour promoting mechanisms. This required gallbladder samples that simultaneously contain non-tumoral (*i.e.* histologically normal) epithelium, low-grade BillN, high-grade BillN and adenocarcinoma, from patients with distinct cancer etiology. Each lesion must be large enough to enable us to analyse the whole transcriptome of several regions of each type of lesion. Samples from two patients were identified in the biobank of the Cliniques Universitaires Saint-Luc: Patient #1 was an 81 year old woman who underwent cholecystectomy to treat cholecystitis; adenocarcinoma was an incidental finding. Patient #2 was a 53 year old man affected with primary sclerosing cholangitis (PSC) whose gallbladder was resected following imaging that revealed a thickening of the gallbladder wall. Pathological diagnoses of non-tumoral epithelium, BillNs and adenocarcinoma were made on H&E-stained sections, and were confirmed by two expert pathologists. Patient #1 displayed two small foci of intestinal metaplasia, and no metaplasia was detected in Patient#2. GeoMx Digital Spatial Profiling (NanoString) [30] was implemented on sections adjacent to the H&E-stained sections to collect whole transcriptome data from 56 epithelial ROIs, each covering approximately 200 epithelial cells of non-tumoral epithelium, BillN and adenocarcinoma ([Table 1](#)). Metaplasia in Patient #1 were too small for spatial profiling. Figure 1 illustrates the spatial distribution of areas in which ROIs were delineated ([Figure 1A](#)), as well as examples of H&E-stained non-tumoral epithelium, BillNs and adenocarcinomas ([Figure 1B](#)). Epithelial ROIs were delineated on sections stained with antibodies which detect markers of the epithelium (panCytokeratin), leukocytes (CD45), and mesenchymal cells (α smooth muscle actin). Nuclei were immunolabeled with anti-Human antigen R antibodies ([supplementary material, Figure S1](#)). The ROIs were subjected to transcriptomic analyses as described in Methods.

Figure 1: Identification and selection of normal epithelium, BillN and adenocarcinoma in samples of human gallbladder. **A** Low magnification view of gallbladder sections. Squares delineate areas in which epithelial ROI were analysed. **B** Illustrative examples of normal epithelium, low-grade BillN, high-grade BillN and adenocarcinoma. ADC, area with adenocarcinomas; H&E, hematoxylin-eosin; HG, area with high-grade BillNs; LG, area with low-grade BillNs; NL, area with normal epithelium



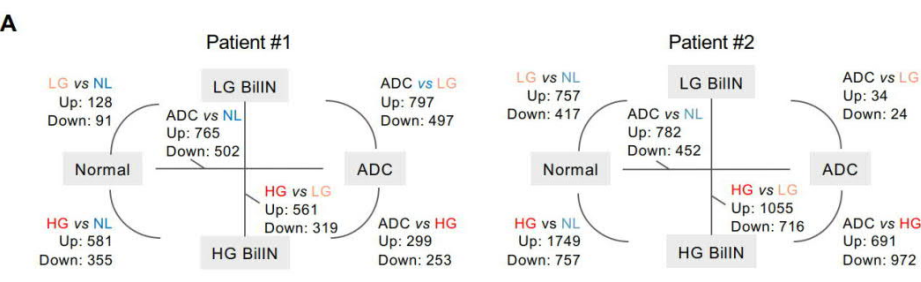
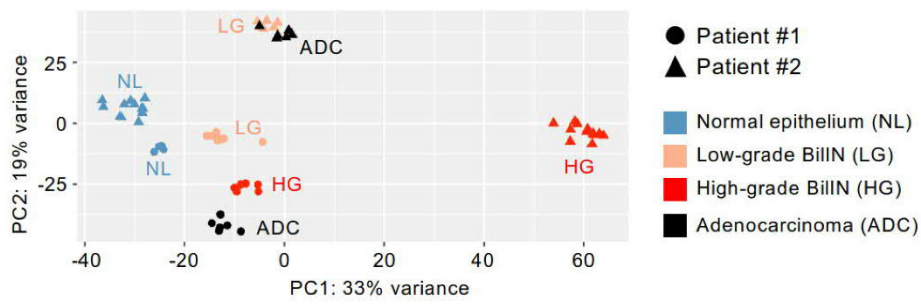
Supplementary Figure 1: Delineation of ROIs for spatial transcriptomic analysis. Illustrative examples of gallbladder sections of Patient #2 with delineation (white line) of epithelial ROIs subjected to spatial transcriptomic analysis. ADC, area with adenocarcinoma; LG, area with low-grade biliary intraepithelial neoplasia (BillN); NI, area with normal epithelium.



Spatial transcriptomic analysis suggests limited intra-patient variability and distinct modes of tumour progression among patients

Principal component analysis (PCA) of the 56 transcriptomes revealed a remarkable clustering of the non-tumoral epithelial samples of the two patients (Figure 2A). ROIs from the same type of lesions clustered together within the same patient, but were separated between patients. In Patient #1, adenocarcinoma ROIs clustered close to high-grade BillN ROIs, whereas adenocarcinomas in Patient #2 appeared closely related to low-grade BillNs. These results were corroborated by the number of differentially expressed genes ($\log_2$ fold change ≥ 1.0 ; $p_{\text{adj}} \leq 0.05$) when cross-comparing all tissue types (supplementary material, Figure S2A). Together, these data revealed that each lesional type displays limited intra-patient variability, but that distinct mechanisms are driving tumourigenesis in the two patients. Moreover, the PCA plot suggested that adenocarcinoma evolved according to a normal $\rightarrow$ low-grade BillN $\rightarrow$ high-grade BillN $\rightarrow$ adenocarcinoma sequence in Patient #1, and according to a normal $\rightarrow$ low-grade BillN $\rightarrow$ adenocarcinoma sequence in Patient #2, with high-grade BillN emerging separately from adenocarcinoma in this patient.

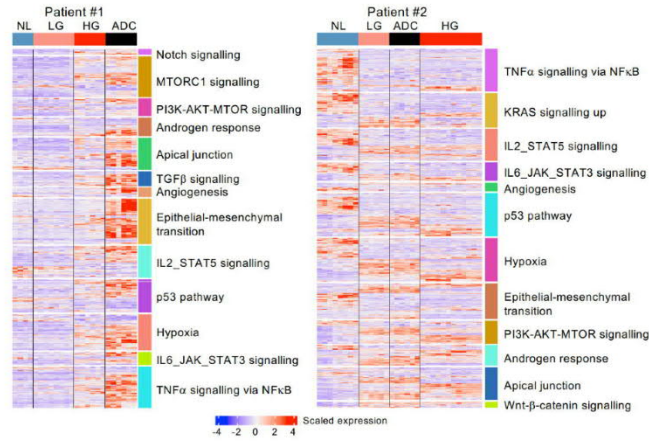
Figure 2A: Distinct modes of tumour progression revealed by spatial transcriptomic analysis. Principal component analysis plot of the whole transcriptome of 60 ROIs comprising normal epithelia, low-grade biliary BiINs, high-grade BiINs and adenocarcinomas.



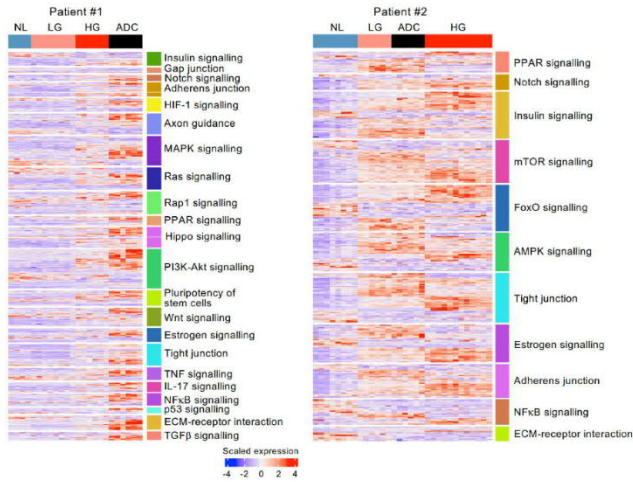
Supplementary Figure 2A: Differential gene expression in spatial transcriptomics: Genes up- or downregulated in cross-comparison of tissue types ($\log_2$ fold change ≥ 1.0 ; $\text{padj} \leq 0.05$).

We next compared the lesions in the two patients and focused on signalling pathways. Using Gene Set Enrichment Analysis (GSEA) [32], we found several enriched signalling pathways when comparing adenocarcinoma and non-tumoral epithelium. Negative or positive enrichment scores reflect enrichment of downregulated or upregulated genes, respectively (Figure 2B). The use of KEGG or HALLMARKS gene sets revealed several pathways that were enriched in both patients, and other pathways that were enriched in only one patient. Heatmaps illustrate genes from the HALLMARKS and KEGG pathway gene sets that are differentially expressed between adenocarcinoma and non-tumoral epithelium in the two patients (Figure 2C and supplementary material, Figure S2B).

Figure 2B : Heatmaps of genes from the HALLMARK gene sets that are differentially expressed between adenocarcinoma and normal epithelium ROIs ($p_{adj} \leq 0.05$).



Supplementary Figure 2B: Heatmap of genes from the KEGG pathway gene sets that are differentially expressed between adenocarcinoma and normal epithelium ROIs in the two patients ($p_{adj} \leq 0.05$). ADC, adenocarcinoma; HG, high-grade biliary intraepithelial neoplasia (BillN); LG, low-grade BillN; NL, normal epithelium.



Galbladder cancer is often associated with mutations in *PI3KCA*, *CTNNB1*, *KRAS*, *TP53*, and *ERBB2* [12-20]. GSEA revealed that PI3K-AKT-mTOR signalling (HALLMARK) is enriched in adenocarcinoma of both patients (Figure 2C), and out of the 38 leading edge genes in Patient

#2, 23 overlapped with the leading edge genes in Patient#1. HALLMARK gene sets are based on coordinately expressed and biologically relevant genes, and identify pathway activation phenotypes [33]. Therefore, the positive enrichment of PI3K-AKT-mTOR signalling reflects activation of the pathway. Further, GSEA suggested enrichment of Wnt signalling in both patients, when considering the KEGG Wnt signalling gene set in Patient #1 and the HALLMARK Wnt- β -catenin gene set in Patient #2 (Figure 2C). However, the two gene sets differ in their composition, leading to different conclusions in the two patients. In Patient #1, Wnt ligands (*WNT7B*, *WNT8A*, *WNT10A*, *WNT11*), receptors (*FZD2*, *FZD5*) and effector (*TCF7L2*) were upregulated in adenocarcinoma as compared to non-tumoral epithelium. Genes induced by Wnt signalling and reflecting activation of a negative feedback loop (*AXIN2*, *GSK3B*) further reveal dynamic activity of the Wnt pathway in this patient (supplementary material, Figure S2C). In contrast, in Patient #2, only 13 genes from the HALLMARK Wnt- β -catenin gene set were significantly enriched. Among these, most genes are not typical for Wnt signalling and belong to pathways with which Wnt signalling crossreacts. *CTNNB1* is upregulated in adenocarcinoma of Patient #2 ($\log_2$ fold change=1.10; $p_{\text{adj}}=8.65 \times 10^{-10}$), in parallel with upregulation of Wnt signalling inhibitors *DKK4* ($\log_2$ fold change=0.86; $p_{\text{adj}}=84.76 \times 10^{-4}$) and *CSNK1E* ($\log_2$ fold change=0.51; $p_{\text{adj}}=1.66 \times 10^{-3}$). Therefore, the analysis of genes of the HALLMARK Wnt- β -catenin gene set does not strongly support that Wnt signalling is active in Patient #2. KRAS signalling differs between the two patients, as evidenced by enrichment of RAS signalling (KEGG) in Patient #1, but downregulation of several KRAS targets within the KRAS signalling up gene set (HALLMARK) in Patient #2 (Figure 2C). Similar to KRAS signalling, the p53 pathway differed between patients. Finally, GSEA did not highlight ERBB signalling. However, we found significant overexpression of EGFR, ERBB2 and ERBB3 in Patient #2, but only overexpression of ERBB2 in Patient #1 (Figure 3A).

Figure 2C: Heatmaps of GSEA enrichment scores using the KEGG pathway and HALLMARK gene sets ($p_{adj} \leq 0.05$). ADC, adenocarcinoma; HG, high-grade BiIN; LG, low-grade BiIN; NES, normalised enrichment score; NL, normal epithelium.

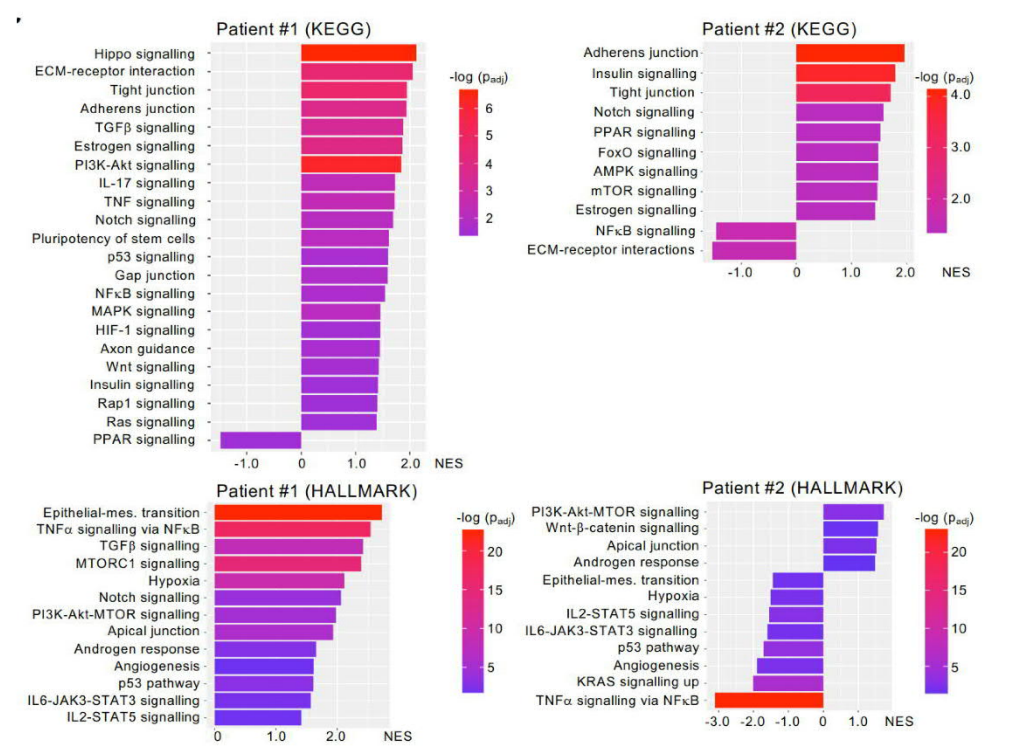
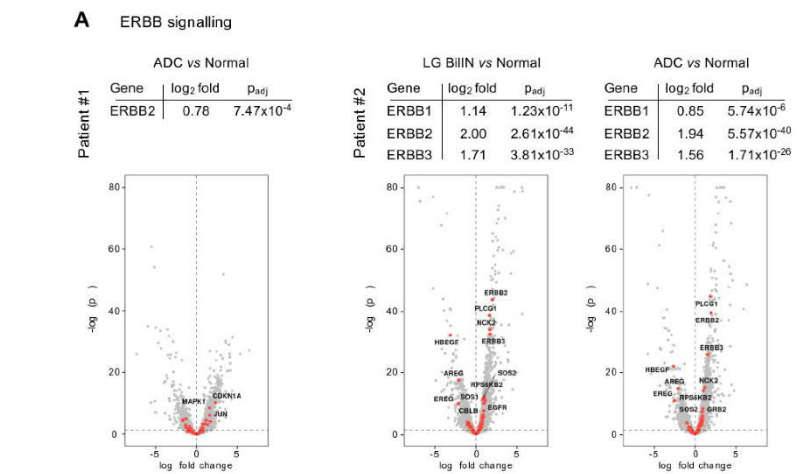


Figure 3A: Distinct modes of signalling pathway activation revealed by spatial transcriptomic analysis. Expression of ERBB receptors and ERBB signalling pathway genes during tumour progression



Although both patients can display enrichment of the same pathway, we noticed that the sequence of enrichment during tumourigenesis may differ among the patients. Indeed, PI3K-AKT-mTOR signalling became enriched in precursor lesions of Patient #1, namely at the low-grade BillIN → high-grade BillIN transition, whereas it became enriched only at the adenocarcinoma stage in Patient #2 (Figure 3B). Other pathways whose enrichment is shared between the patients may in contrast display a similar sequence of enrichment. Indeed, androgen response and estrogen signalling became enriched at the precursor-to-adenocarcinoma transition (Figure 3B). Notch signalling was also enriched in adenocarcinoma of both patients, and the enrichment was only significant when comparing non-tumoral epithelium and adenocarcinoma, not when comparing the precursor to adenocarcinoma transitions. This likely reflected a progressive enrichment throughout the tumourigenic process, without significant jumps between lesional states. Moreover, comparing the expression of leading edge genes in the enriched Notch pathway also revealed interesting differences such as the strong upregulation of *NOTCH3* in Patient #1 ($\log_2$ fold change=2.05; padj= 5.2×10^{-11}) and more modest upregulation of this gene in Patient #2 ($\log_2$ fold change=0.77; padj= 1.5×10^{-3}) (Figure 3C).

Figure 3B: Sequence of enrichment of signalling pathways during tumour progression as determined by GSEA using KEGG pathway and HALLMARK gene sets

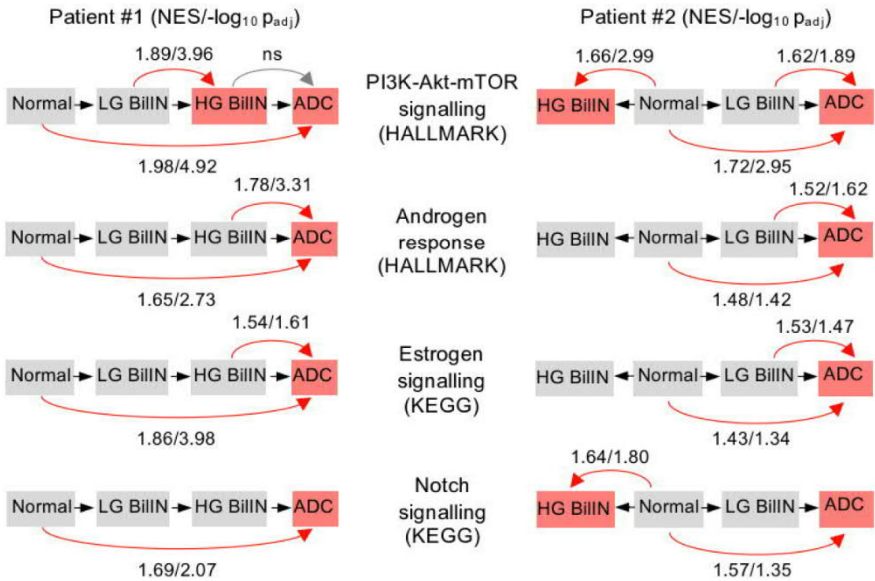
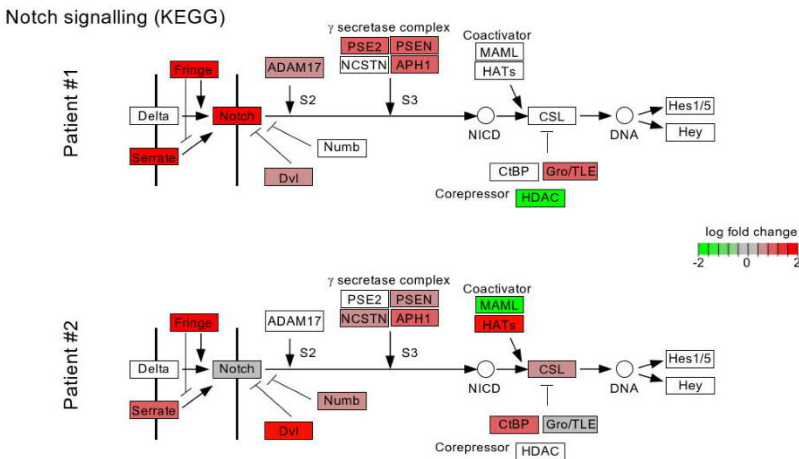


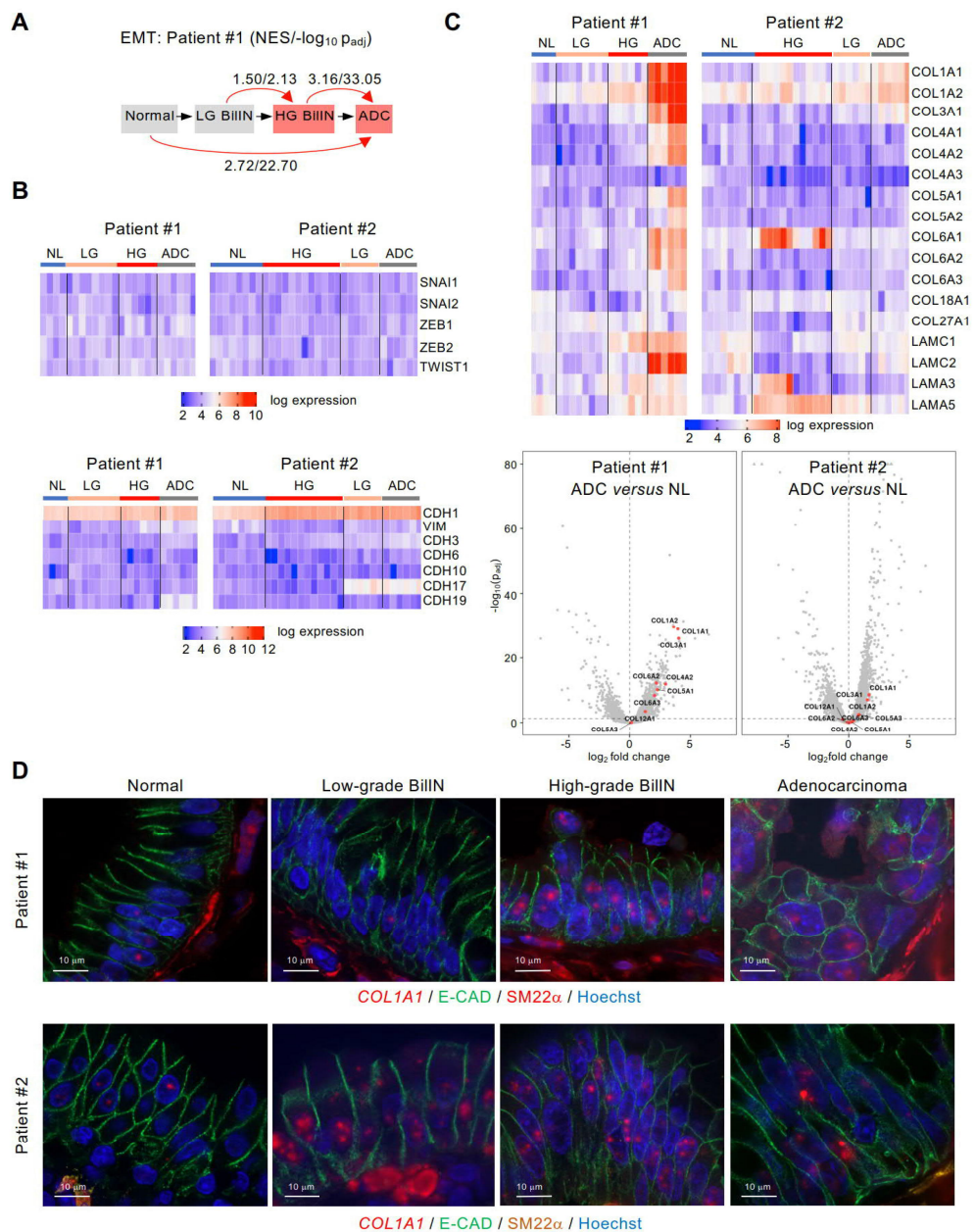
Figure 3C: Differential expression of Notch signalling genes (KEGG) between adenocarcinoma and normal epithelium. ADC, adenocarcinoma; HG, high-grade BiIN; LG, low-grade BiIN; NES, normalised enrichment score; ns, non-specific



Spatial transcriptomic analysis identifies hybrid epithelial and mesenchymal states

Nepal and coworkers considered the hallmark "epithelial-mesenchymal transition (EMT)" as indicative of poor prognosis [20]. In Patient #1, the corresponding HALLMARK gene set has the highest enrichment score when comparing adenocarcinoma with non-tumoral epithelium (Figure 2B-C). The sequence of EMT enrichment is shown in Figure 4A. No similar enrichment was found in Patient #2. Transcription factors typical for EMT and *CADHERINS* showed no significant differential expression during tumour progression in either patient (Figure 4B). In contrast, extracellular matrix-coding genes were significantly upregulated (Figure 4C). To support the latter data at the histological level, we resorted to RNAscope *in situ* hybridisation. We detected rare mRNAs coding for *COL1A1* in non-tumoral epithelia of the two patients. Strong induction of *COL1A1* was detected in high-grade BiIN of Patient #1, but also in low-grade BiINs of Patient #2 (Figure 4D). We concluded that both patients displayed criteria of hybrid epithelial and mesenchymal states. The marked EMT in Patient #1 suggests that the adenocarcinoma belongs to the poor prognosis category.

Figure 4: Partial epithelial-mesenchymal transition during tumour progression. **A** Enrichment sequence of EMT (HALLMARK) in Patient #1 demonstrates enrichment throughout tumorigenesis. **B** Gene expression heatmaps of EMT-promoting transcription factors, and of VIMENTIN and CADHERINS shows little or no variation during tumorigenesis. **C** Heatmap and volcano plots showing COLLAGEN and LAMININ gene expression in the two patients. **D** RNAscope in situ hybridisation demonstrates induction of COL1A1 mRNA (red dots) starting in high-grade BiINs in Patient #1 and in low-grade BiIN of Patient #2. Tissue sections were immunostained to mark epithelial cells (E-CADHERIN; E-CAD), nuclei (Hoechst), and mesenchymal cells (Smooth muscle protein 22 α ; SM22 α). ADC, adenocarcinoma; HG, high-grade BiIN; LG, low-grade BiIN; NES, normalised enrichment score; NL, normal epithelium



SEMAPHORIN4A downregulation promotes tumour progression

Our GSEA data uncovered axon guidance signalling as a potential driver of tumour progression in Patient #1 (Figure 2C). Axon guidance genes, including *SEMAPHORIN/PLEXIN* ligand-receptor pairs, were enriched in Patient #1 adenocarcinomas, but not in Patient #2 (supplementary material, Figure S3). *SEMA4A* was downregulated in the adenocarcinomas of both patients, and this was noticed already at the precursor stages (Figure 5A). The involvement of *SEMA4A* in gallbladder cancer is unexplored, but *SEMA4A* loss-of-function mutation in familial colorectal cancer type X was found to promote cancer development, thereby revealing a tumour suppressor role for *SEMA4A* [34, 35]. This prompted us to investigate the role of *SEMA4A* in gallbladder cancer development.

Supplementary Figure 3A: Expression of axon guidance genes. Axon guidance pathway (KEGG) enriched in Patient #1 during tumour progression (adenocarcinoma versus normal)

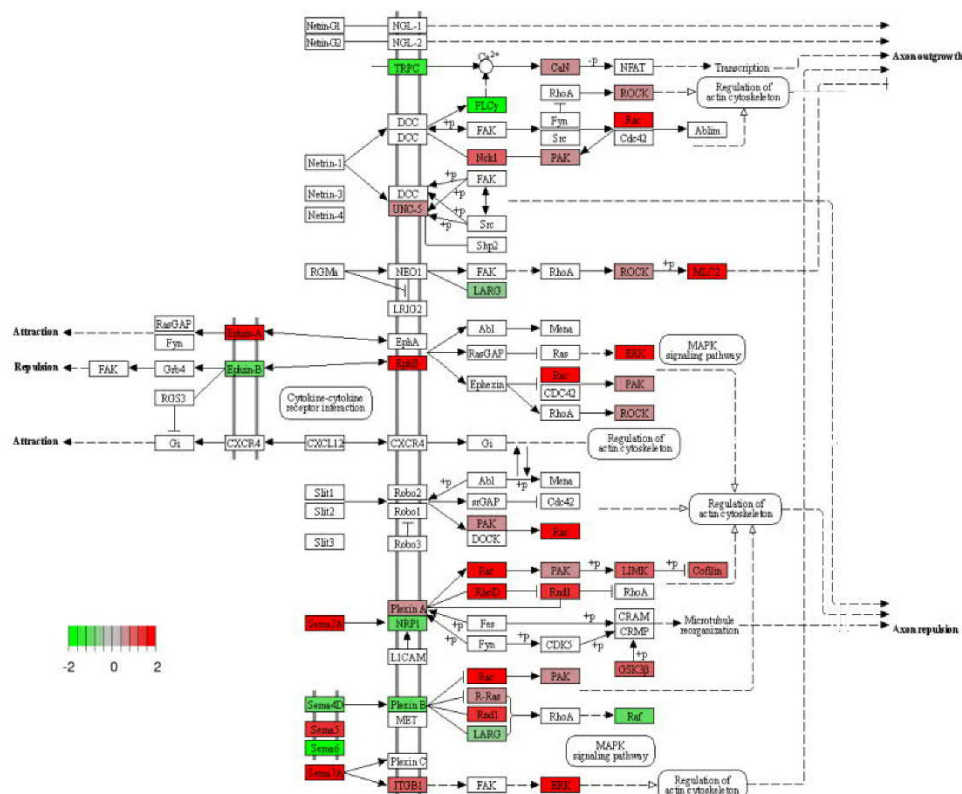
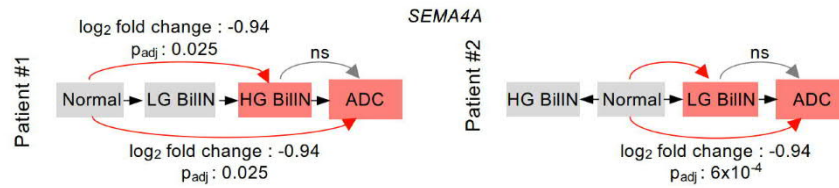
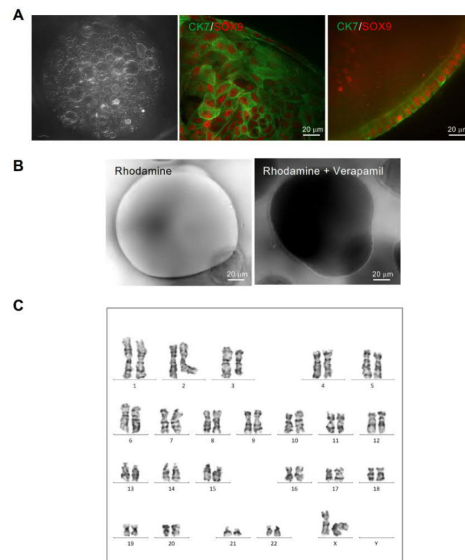


Figure 5A: *SEMA4A* gene expression is reduced during gallbladder cancer progression. ADC, adenocarcinoma; HG, high-grade BillN; LG, low-grade BillN; NES, normalised enrichment score; NL, normal epithelium; ns, non-specific



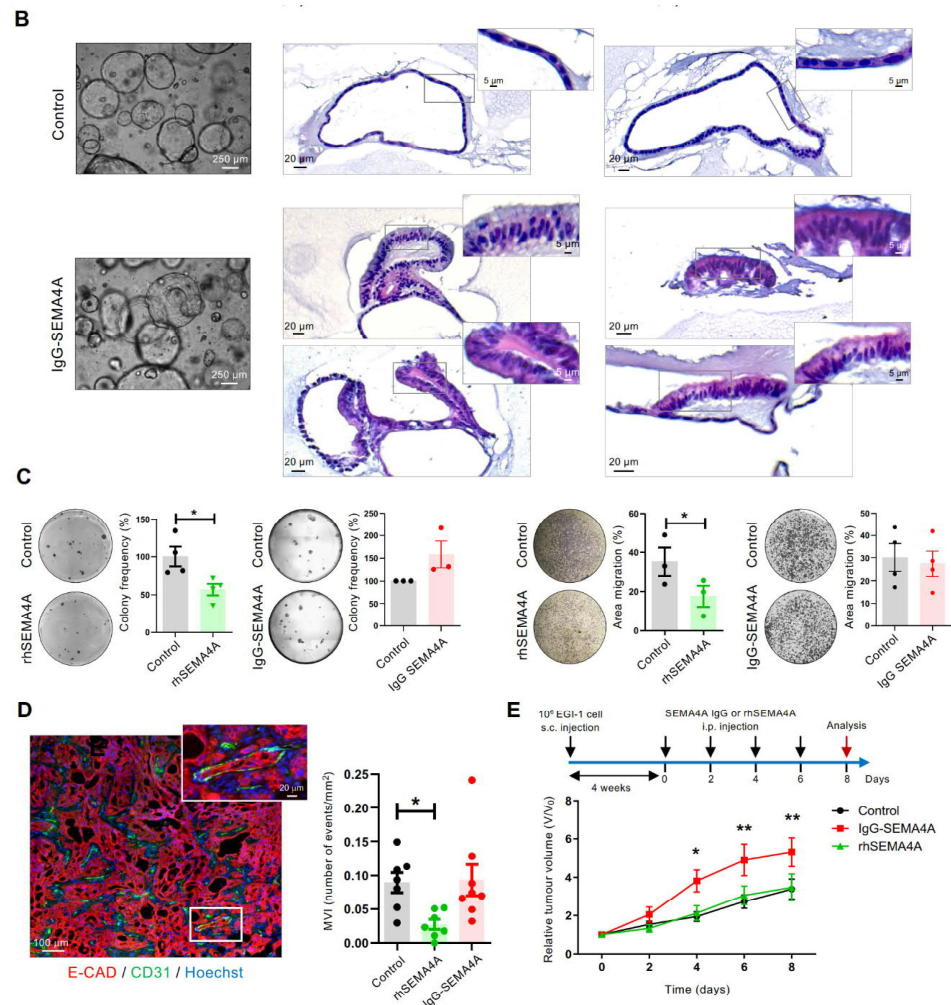
We first generated organoids from gallbladder epithelium and selected a line which displayed no karyotypic anomalies. It expressed biliary-specific markers and exhibited biliary transport functions ([supplementary material, Figure S4](#)). To mimick the downregulation of *SEMA4A* observed in our transcriptomic analyses, we incubated the organoids for 3 days with a blocking anti-*SEMA4A* IgG antibody. We found no change in cell proliferation, but observed local areas of pseudostratification of the epithelium in a subset of organoids ([Figure 5B](#)). The histology of those areas was reminiscent of BillN, indicating that inhibiting *SEMA4A* impacts cell polarisation.

Supplementary Figure 4: Characterization of gallbladder organoids. **A** Gallbladder organoids were grown in Matrigel and visualised using light microscopy (left panel). Immunostaining revealed expression of the biliary-specific proteins CYTOKERATIN 7 (CK7) and SRY-related HMG box transcription factor 9 (SOX9). **B** Intraluminal accumulation of Rhodamine (100 μ M) that was added to the organoid medium was inhibited by Verapamil (10 μ M), demonstrating the functionality of MDR1-mediated transport. **C** Normal karyotype of the selected organoid line.



We next determined if SEMA4A had additional tumour suppressor properties. Since the organoid lines were not able to induce tumour formation after subcutaneous injection in immunodeficient NSG mice, we used the human extrahepatic cholangiocarcinoma cell line EGI-1. *In vitro*, clonogenic and transwell migration assays demonstrated that adding rhSEMA4A to cultured EGI-1 cells reduced their clonogenicity and migration (Figure 5C). Blocking anti-SEMA4A IgG antibody slightly but not significantly increased colony formation, and did not impact cell migration (Figure 5C). *In vivo*, subcutaneous injection of EGI-1 cells in immunodeficient NSG mice resulted in the formation of tumours. Consistent with the decreased migration induced *in vitro* by rhSEMA4A, intraperitoneal administration of rhSEMA4A resulted in a significant reduction of microvascular invasion in EGI-1 cell-derived tumours (Figure 5D). Anti-SEMA4A IgG antibody had no effect on microvascular invasion in the tumours. Recombinant SEMA4A did not impact tumour growth. In contrast, blocking IgG anti-SEMA4A antibody accelerated growth at the earliest stages of tumour growth to progressively reach a plateau (Figure 5E). We conclude that SEMA4 can control tumour progression by impacting polarity, clonogenicity and migration of cells.

Figure 5B-5E SEMA4A displays tumour suppressor properties. **B** The epithelium of gallbladder organoids treated with blocking anti-SEMA4A IgG antibody displays focal areas of pseudostratification. This effect was monitored in two experiments out of four. **C** rhSEMA4A reduces clonogenicity and migration of cultured EGI-1 cells, whereas anti-SEMA4A IgG antibody had little or no effect. Data show means $\pm$ SEM; $n=3$ or 4; statistical significance was calculated by applying a paired t-test (*, $p<0.05$). **D** Microvascular invasion (MVI) is illustrated in subcutaneous EGI-1 cell tumours following intraperitoneal injection of rhSEMA4A or of blocking IgG SEMA4A antibodies, according to the timing shown in panel E. The graph shows that rhSEMA4A reduces the number or MVI events in EGI-1 cell tumours. One-way ANOVA was used to compare means (*, $p<0.05$). **E** Growth of subcutaneous EGI-1 cell tumours following intraperitoneal injection of PBS (control), blocking anti-SEMA4A IgG antibody, or rhSEMA4A. $n=10$ (control), 8 (IgG SEMA4A) and 7 (rhSEMA4A). Relative tumour volume and SEM are plotted. Differences between groups were evaluated by performing a two-way Analysis of Variance (two-Way repeated measures ANOVA) with Bonferroni correction (*, $p<0.05$; **, $p<0.01$). For further statistical validation, a random intercept- random slope model with continuous time was fitted. This showed a significant interaction between the time and group effect ($p=0.03$), in particular, the contrast between SEMA4A IgG and control is significant ($p=0.048$) but not that between control and rhSEMA4A ($p=0.95$).



DISCUSSION

Earlier mutational profiling provided evidence that adenocarcinoma development may be BillIN-dependent or -independent [11]. Here, using GeoMx technology we confirmed and extended these findings at the transcriptional level and showed that lesions displayed little inpatient variability. There was unfortunately not enough tissue material to characterise the genomic profile of the lesions.

In Patient #1, ROIs from a same type of lesion were often located at a rather short distance from each other, except for adenocarcinoma ROIs which were more scattered throughout the tissue sample. In Patient #2, high-grade BillIN ROIs were close to each other, but low-grade BillIN, adenocarcinoma and non-tumoral epithelium ROIs were significantly dispersed ([Figure 1A](#)). Still, in spite of the scattering within the tissue, the transcriptomic profile of lesions belonging to the same histological type showed limited intra-patient variability. Such transcriptomic homogeneity likely reflects that cells from a same type of lesion proliferated in a similar environment and with limited accumulation of new mutations. Clonal analysis of gallbladder cancers revealed subclonal diversification [36], and this may lead us to anticipate significant intra-patient variability in the adenocarcinoma lesions studied in our work. However, our patient samples contained all lesional types on the same tissue sections, suggesting that cancer lesions had not enough time to accumulate genomic lesions, invade the tissue and produce subclones.

The neighbourhood of low-grade BillIN, high-grade BillIN and adenocarcinoma which may occur in pathological samples, leads us to surmise that the epithelium undergoes a normal epithelium → low-grade BillIN → high-grade BillIN → adenocarcinoma histogenic sequence. *A contrario*, the transcriptomic profile of Patient #2 strongly suggests that adenocarcinoma derived from low-grade BillIN, not from adjacent high-grade BillINs. This contrasted with Patient #1 whose adenocarcinoma ROIs were closely related to high-grade BillINs. We excluded that adenocarcinoma in Patient #2 corresponded to low-grade BillINs extending in Rockitansky-Aschoff sinuses. In Patient #2, only 58 genes were 2-fold up- or downregulated when comparing low-grade BillIN and adenocarcinoma, revealing that low-grade BillIN may be at high risk for evolution towards invasive cancer.

Many signalling pathways were activated during tumour progression and several were common between the two patients. However, the sequence of pathway activation differed between patients, some of the common pathways being activated at the BillIN stage in one patient, but only in the adenocarcinoma cells in the other patient. Therefore, our work suggests that various combinations of pathway activations may end up yielding cancer, no specific pathway or combination of pathways being responsible for transition from one stage to the other.

The HALLMARK gene set "Inflammatory response" was enriched in adenocarcinomas of both patients (not shown), reflecting their common chronic inflammatory background. Still, the tumour etiology differed in Patients #1 and #2, with Patient #2 being affected with PSC, a

disease with high incidence of adenocarcinoma [37]. The adenocarcinoma in Patient#2 was mucosecreting (Figure 1B), unlike the carcinoma in Patient #1. The mutational profile of cholangiocarcinoma in PSC is heterogeneous and affects genes similar to those in non-PSC associated cholangiocarcinoma, the most frequently mutated being *TP53*, *KRAS*, *PI3KCA* and *GNAS*. In low-grade and high-grade dysplastic lesions, loss or amplifications of several genes, as well as mutations in *ERBB2* and *TP53*, can already occur [38, 39]. Our work extend these data at the transcriptomic level and highlight that low-grade BillIN can be very closely related to adenocarcinoma.

EMT is a phenotypic continuum during which epithelial cells evolve to a mesenchymal state via multiple transitional or hybrid states [40]. It involves disruption of polarity and intercellular adhesion, changes in the interaction between cells and extracellular matrix, and increased migratory properties [41, 42]. Our RNAscope analysis of *COL1A1* expression demonstrated that signs of EMT are detectable early on in epithelial cells during tumour progression, reflecting the emergence of hybrid epithelial-mesenchymal states.

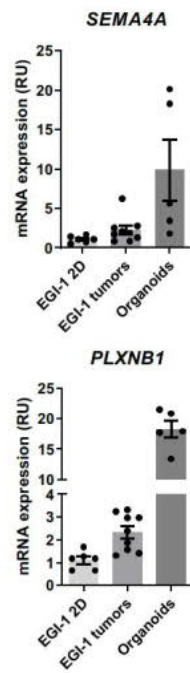
SEMA4A is a tumour suppressor in colorectal cancer [34, 35]. Here we found that it is downregulated in the two patients during gallbladder tumour progression, starting at the BillIN stage. Gallbladder organoids expressed *SEMA4A* and its receptor *PLEXINB1* (*PLXNB1*), and the levels of *SEMA4A* varied considerably (supplementary material, Figure S3B), likely explaining the variable pseudostratification of the gallbladder organoids when treated with blocking IgG antibody (Figure 5B). Also, the low levels of *SEMA4A* and *PLXNB1* in cholangiocarcinoma EGI-1 cells, as compared to organoids derived from normal gallbladder epithelium, fit with the notion that *SEMA4A* is repressed in biliary cancer cells and with our observation that anti-SEMA4 blocking antibodies have limited or no effect on clonogenicity and migration of EGI-1 cells *in vitro*. *In vivo*, we detected a higher level of *SEMA4A* in EGI-1 cell-driven tumours than in *in vitro* cultured EGI-1 cells (supplementary material, Figure S3B). We excluded that this results from *SEMA4A* production by tumour-invading mouse cells, as our

PCR primers were designed to specifically detect human SEMA4A. Inhibiting this *in vivo* production of SEMA4A enabled us to monitor growth-promoting properties of anti-SEMA4 blocking antibodies. How these anti-SEMA4A antibodies promote EGI-1 cell-derived tumour growth remains unclear. Indeed, our data show that inhibiting SEMA4A accelerates tumour growth during 4 days. This effect slows down to reach a plateau (Figure 5E), and at the plateau stage we noticed a slight but not significant increase in proliferation rate, as evidenced by immunostaining for phospho-Histone H3 (supplementary material, Figure S3C). We hypothesize that anti-SEMA4A antibodies promoted proliferation mainly during the first 4 days of treatment. Interestingly, rhSEMA4 did not impact tumour growth, but decreased microvascular invasion, suggesting that reduction of SEMA4 promotes metastasis. The signalling pathways mediating the effects of SEMA4A on migration, polarity and potentially proliferation deserve further investigation. Further studies will determine how frequently SEMA4A is repressed at early stages of gallbladder cancer and whether understanding its pathway may lead to identify biomarkers of early diagnosis of gallbladder tumours.

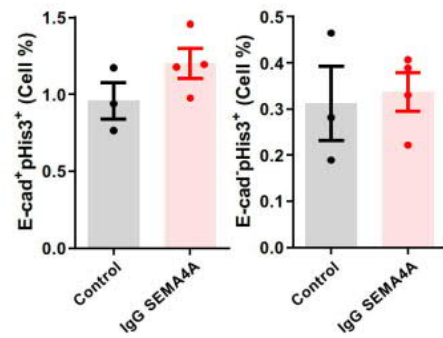
In conclusion, our spatial transcriptomic analysis reveals that precursor and cancer lesions display limited intra-patient variability during gallbladder cancer progression and supports that tumourigenic mechanisms are patient-specific. They also underscore that low-grade BillNs may be at high risk for developing to cancer and should ideally be characterised by gene expression profiling. Further, repression of SEMA4A may contribute to tumour progression.

Supplementary figures 3B and 3C : Expression of SEMAPHORIN 4A (SEMA4A) and its receptor PlexinB1 (PLXNB1) in cultured EGI-1 cells, EGI-1-derived subcutaneous tumours, and cultured gallbladder organoids, as measured by RT-qPCR. SEMA4 and PLXNB1 primers are human-specific and do not detect potential expression in murine cells colonising EGI-1-cell derived tumours. **C** Percentage of E-CADHERIN (E-CAD)-positive cells and E-CADHERIN-negative cells expressing phospho-Histone H3 (pHis3) in EGI-1-cell-derived tumours at day 8 of treatment with PBS (= control) or blocking anti-SEMA4A IgG antibodies. NES, normalised enrichment score.

B



C



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AUTHOR CONTRIBUTION

S.P., F.M.-N., A.L., M.K., L.G. and F.L. designed the study; S.P., F.M.-N., A.L., S.C., S.A., C.H., S.T., acquired data; S.P., F.M.-N., A.L., L.D., N.L., C.S., M.K., L.G. and F.L. analysed and interpreted data. S.P., F.M.-N., A.L., L.D., N.L., L.G. performed statistical analyses. S.P., F.M.-N. and F.L. drafted the manuscript. All authors read and approved the final paper.

DATA AVAILABILITY

The raw dataset generated from GeoMx DSP analysis during the current study is available in supplementary material.

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Supplementary material

Table I. Number of ROIs subjected to spatial transcriptomic analysis.

	Patient #1	Patient #2
Non-tumoral	4	8
Low-grade BillN	8	6
High-grade BillN	6	12
Adenocarcinoma	6	6

Supplementary Table S1. List of primary and secondary antibodies

Primary antibody	Host	Company and reference	Dilution	
			IF	RNA scope
CD31	Rat	Dianova #DIA-310	1:100	
Col1A1	Rabbit	Cell Signaling #39952S	1:200	
E-CADHERIN	Mouse IgG2	BD #610182	1:300	1:100
SM22 α	Rabbit	Proteintech #10493-1-AP		1:50

Secondary antibody	Host	Company and reference	Dilution	
			IF	RNA scope
Alexa Fluor 488 anti-rat	Donkey	ThermoFisher Scientific #A-21208	1:500	
Alexa Fluor 488 mouse igG2a	Goat	ThermoFisher Scientific A-21131	1:500	1/500
Alexa Fluor 594 anti-mouse	Donkey	ThermoFisher Scientific #A-21203	1:500	
Alexa Fluor 594 anti-rabbit	Donkey	ThermoFisher Scientific A-21207	1:500	1/500

Supplementary Table S2 – List of primers used for RT-qPCR

Target		Forward Primer	Reverse Primer
RPL19	H	5'-CGAATGCCAGAGAAGGTCAC-3'	5'-CCATGAGAATCCGCTTGTTT-3'
SEMA4A	H	5'-AATGGCTCCCTCTTGCTGAT-3'	5'-GACTGGAGGTGTTGCTCTCT-3'
PLXNB1	H	5'-CCTCACCTTTGATGGGACCT-3'	5'-GCACATCACACCAGAACCAG-3'

IV. General discussion

My thesis raises questions related to both the selected methods to achieve my goals, and the results that were obtained. These questions are discussed in the following two sections.

IV.1 Methodology: strengths, limitations and perspectives

To address the question of the thesis, I used transgenic mouse models, spatial transcriptomic analyses of human samples, organoids and cancer cell lines. These different methods have their strengths and weaknesses.

We succeeded to generate murine ICCAs and IPNBs in OpnCre^{ER} LSL-Kras^{G12D} mice fed a DDC diet whereas several attempts to induce GBC using a Ck19Cre^{ER} system failed. Despite these last negative results, conditional transgenic mouse models provide temporal and spatial control of gene expression and remain a good approach to study tumor development in vivo. Indeed, in these models, tumors grow in vivo, in situ and interact with the microenvironment. Moreover, to investigate tumor initiation mechanisms, we had to consider premalignant/precursor lesions and the best way to prove their malignant potential is to show that these lesions evolve to cancer. In that context, mouse models offer the opportunity to investigate stepwise progression from precursor to malignant state, as was performed when analyzing OpnCre^{ER} LSL-Kras^{G12D} mice fed a DDC diet. The histology of the lesions cannot be studied at different time points within the same individual, but the reproducibility of the phenotype supports the validity of the proposed histogenic pathway from IPNB to iCCA. An optimal mouse model of tumor development requires expression of mutations at a physiological level, which is what we aimed for by expressing Kras^{G12D} from the endogenous Kras locus in OpnCre^{ER} LSL-Kras^{G12D} mice. The drawback of such approach is the slow speed of tumor development (25-30 weeks of DDC diet to obtain iCCA), which is cumbersome in the planning of experiments. A risk associated with mutant gene induction in vivo is the potential elimination of cells bearing the mutation. Indeed, apical extrusion allows normal epithelial cells to actively eliminate newly mutated cells (88) and I detected potentially extruded cells in the Ck19Cre^{ER} LSL-p53^{R172H} LSL-Rosa26^{APOBEC3B-IRES-eYFP} and Ck19Cre^{ER} LSL-Rosa26^{APOBEC3B-IRES-eYFP} mice during our

attempts to induce tumor formation in gallbladders. We also considered the possibility that the expression of a mutation in a normal cell could be toxic for the cell and induce cell death.

Because the transgenic mouse models aimed to induce GBC did not generate tumors, I developed a spatial transcriptomic analysis of human samples containing simultaneously the precursor and cancer lesions. The GeoMx Digital Spatial Profiler from Nanostring was selected to study the whole transcriptome of precursor lesions (BillIN low grade and BillIN high grade) and GBC in 2 patients. The high spatial resolution of the technique enabled us to accurately target the selected regions of interest i.e. the epithelium of precursor lesions and GBC. At the time when our experiments were performed, GeoMx digital spatial profiling was the only technique compatible with formalin-fixed paraffin-embedded tissue. This was required, first, because BillIN lesions are asymptomatic and diagnosed on the resection specimen, after the surgery, which makes frozen tissues of BillIN unavailable. Second, formalin-fixed paraffin-embedded tissue enabled us to accurately delineate the studied regions with help of high quality hematoxylin and eosin staining on an adjacent section. GeoMx digital spatial profiling offered several advantages over the laser capture microdissection that was used to investigate our mouse model of iCCA, namely high spatial resolution, no damage of the borders of the sample by the laser, compatibility with immunostaining to identify specific structures, no requirement of specific slides. However, a limitation of our study was the need to delineate regions of interest that included a minimum of 200 cells located in a 750 x 650 μm rectangle. Current technologies enable single cell analyses.. In contrast to laser capture followed by RNA sequencing, GeoMx does not provide RNA sequence information on the studied samples and is not informative regarding potential mutations. Finally, despite the high spatial resolution of the GeoMx Digital Spatial Profiler and the accuracy of its selection, the sections submitted to the transcriptomic analysis were 5 μm thick. This means that some transcriptomic data could have been obtained from other cell types (for example, fibroblasts) or other epithelial cells that do not present the histological criteria defining the region of interest.

Most studies investigate a limited number of lesions in a rather large cohort of patients. We favored the analysis of a high number of regions of interest within a same patient, and this was performed on two patients with distinct natural history

of the disease. Admittedly, the high costs and low number of patient samples harboring simultaneously low and high grade BillNs and GBC prevented us to study a larger cohort of patients. To overcome this limitation, I focused my transcriptomic analysis on biological events shared by the two patients and potentially driving tumor progression regardless of the clinical context. Unfortunately, combined analysis of DNA and RNA was impossible using the GeoMx Digital Spatial Profiler. Manual microdissection of the same regions of interest on an adjacent section to extract DNA to detect potential mutations was not technically feasible given the low amount of DNA that could be extracted. This prevented us to determine the evolutionary trajectory of gallbladder lesions using DNA information, as was performed by Lin et al. (67).

The question of a possible contamination of the transcriptomic data by non epithelial cells arose when I detected an upregulation of fibrillar collagen during tumor progression. Indeed, fibrillar collagens normally belong to the extracellular matrix and are secreted by fibroblasts. In line with the spatial transcriptomic data, I confirmed the expression of COL1A1 mRNA in epithelial cells on tissue sections adjacent to those subjected to Nanostring. The variable expression of COL1A1 detected under the microscope reflected the upregulation found in the spatial analyses. The COL1A1 protein was also detected by immunohistochemistry (antibody anti-Col1a1 Rabbit anti-human igG, Cell signaling 39952) in a subset epithelial cells from GBC areas.

Principal component analyses of the RNAseq data from the mouse model of ICCA and of the gallbladder spatial transcriptomic analyses was key in our interpretation of the filiation of IPNB to ICCA and of BillNs to GBC. Distinct lesions that are closely related from a transcriptomic point of view likely originate from each other. However, we can only suggest this filiation and a demonstration would require tracing experiments. While such lineage tracing experiments are theoretically feasible in mice, they are not doable in humans. Therefore, the bioinformatic analyses of the data provide us with a statistically significant view of pathophysiological phenomena, which bears the risk of not necessarily reflecting biologically significant phenomena. Bioinformatic analyses of the data also enable to correlate differential expression of genes or pathways with the development of specific histological lesions. Yet, correlation does not mean causality, meaning that experimental validation of proposed causal relationships remains mandatory. This

is what we tried to achieve while focusing on SEMA4A, and which ideally should be extended to investigate the potential tumor promoting role of other pathways highlighted in our analyses.

Cancer cell lines and non-tumoral gallbladder organoids were used during my thesis to test functional hypotheses suggested by transcriptomic data. We used EGI-1 cells, a human cholangiocarcinoma cell line isolated from an advanced biliary tract cancer from a 52 years old German patient in 1982. The accurate subtype of biliary tract cancer (iCCA, eCCA or GBC?) remains unclear and the use of the name “cholangiocarcinoma” in some databases does not exclude the diagnosis of GBC. Another limitation of this cell line is its unclear genomic status, since the cells are cultured for years and may have accumulated additional mutations. Still, they are a biological system enabling to test working hypotheses in a cost-effective way. Tumor-derived organoids are an alternative, yet confronted with other limitations such as biological variability.

We derived non-tumoral gallbladder organoids from patients affected with gallstone disease. The aim was to develop a model of normal cholangiocytes and to test hypotheses derived from the spatial transcriptomic analyses in order to induce different steps of tumor development starting from normal cells. Four organoid lines were developed and grown in Matrigel according to the protocol described by Rimland et al. (89). The four lines displayed cholangiocyte features but differed at the genomic level. Indeed, 1 line showed a suspicion of trisomy 20 and a 16q deletion. In another line, using whole genome sequencing we detected an ERBB3^{R675G} missense mutation. This mutation is not functionally characterized (90) and was found in 4% of the cells. Whether the mutation was present in the patient or appeared during the culture is unknown. Genomic differences between organoid lines are expected, yet how such differences should be taken in account when studying tumorigenesis remains problematic.

Also, unlike normal gallbladder epithelium, organoid cells proliferate as a result of R-spondin-mediated stimulation of the Wnt/ β -Catenin pathway (91). Several lines of evidence suggest the involvement of the Wnt/ β -Catenin pathway in gallbladder carcinogenesis. β -catenin mutations were found in BillIN (67) and hypermethylation of a Wnt inhibitor (WIF1) was detected during gallbladder carcinogenesis (69). The organoid culture medium contains B27 and N2

supplements, and both of them contain progesterone. Yet, female sex is a risk factor of GBC. NBL1, a bone morphogenic protein antagonist, was upregulated during gallbladder carcinogenesis in patient 2 following our spatial transcriptomic experiment and the culture medium of our organoids also includes Noggin, another inhibitor of several bone morphogenic protein that belong to the TGF β superfamily (92). Considering the consequences imposed by the culture medium, organoids derived from non-tumoral gallbladders may be closer to a transforming epithelium than to a normal epithelium. Still, when injected subcutaneously in immunodeficient mice, the organoids did not induce tumor formation, suggesting that they are not tumorigenic. In the same lines, non-tumoral gallbladder organoids treated with increased concentrations of estrogens, EGF, bile acids and TGF β inhibitors (in an attempt to reproduce the enrichment of the corresponding signaling pathways found in our spatial transcriptomic data) did not generate tumor after subcutaneous injection in immunodeficient mice. This means that these experimental conditions were not able to induce tumor formation.

To conclude, in vivo models enabled us to investigate tumor progression in ICCA, and spatial transcriptomic approaches generated a significant amount of new knowledge on GBC tumorigenesis. Unfortunately, none of the selected in vitro methods enabled us to satisfactorily reproduce the initial stages of tumor formation. It is likely that a better combination of genomic and transcriptomic data from patient samples would have provided essential information for recapitulating GBC tumorigenesis. An important issue that was not considered in our study is the importance of the micro-environment which should be included in future work. Finally, since the concept driving the research project was to find targets to improve prophylaxis, future work should combine molecular analyses with epidemiological studies.

IV.2 Results

IV.2.1 Intrahepatic cholangiocarcinoma

OpnCre^{ER} LSL-Kras^{G12D} mice fed a DDC diet generated iCCA. We could not use MUC1 (EMA) staining to distinguish small and large duct type iCCA because the normal murine cholangiocytes displayed an apical staining of MUC1 (EMA) along the entire biliary tree. However, our data show mass forming iCCA, and large duct type iCCA usually presents in humans with periductal infiltrating growth pattern alone or in combination with a mass-forming pattern. I propose that the early cancer lesions correspond to the periductal infiltrating part of a large duct type iCCA that finally gives rise to a major mass forming tumor. Therefore, the iCCAs induced in our mouse model recapitulate very well human iCCA pathology. Still, the major weakness is the lack of distant metastasis, lymph node metastasis and perineural invasion which could have demonstrated the malignant potential of these tumors. One possible explanation is that the development of metastases requires prolonged DDC treatment.

Based on our analysis of the histological lesions occurring in DDC-fed OpnCre^{ER} LSL-Kras^{G12D} mice we propose a model of tumor progression from IPNB to early stage cancer and full blown iCCA. The observation of histological transitional zones between IPNB and early stage cancer served as an argument for a potential filiation from IPNB to cancer. This possible filiation between IPNB and iCCA is in line with transcriptional data showing that the transcriptomes of IPNB and iCCA are closely related. Also, tumor progression is associated with TGF β inhibition (23, 39) in CCA as in our model where p-SMAD2 is expressed in IPNB but not in CCA. Also, the papillary proliferation detected in the lumen of the bile ducts remains intraluminal and resembles human IPNBs. The expansive and disorganized growth pattern of the glandular proliferation described as early stage cancer supports their invasive nature.

However, our claim to have developed a mouse model that recapitulates formation of iCCA precursor lesions and their evolution toward malignancy requires care. First, we have not formally proven the filiation between the lesions by means of lineage tracing. Our transgenic model OpnCre^{ER} LSL-Kras^{G12D} LSL-Rosa^{YFP} (exposed to DDC) does not allow to answer to that question because the YFP protein is expressed in most intrahepatic cholangiocytes whatever their location and

subsequently in the 3 subtypes of lesions (IPNB, early cancer and ductular proliferation). Rainbow mice might represent an option as it might visualise a clone of cells and its expansion. (93, 94). Second, histological transitions between IPNB and early stage cancer lesions could result from an invasion of the portal tract and the bile duct by the iCCA with subsequent intraductal growth. This cannot be excluded but seems less likely because more inflammation would be expected around the IPNB epithelium targeted by invasion by iCCA. Third, the link between early stage cancer lesions and full blown iCCA was not sufficiently investigated to demonstrate the malignant potential of early stage cancer lesions. Staining of the basal membrane on tissue sections and illustration of its disruption could contribute to a more complete description of the lesions.

Our data support a role for Tensin 4 (TNS4) in iCCAs tumor progression like it does in hepatocellular carcinoma (95). We note the variability of some of our experiments, namely in the size of the EGI-1 and TNS4-EGI-1 xenografts. Moreover, the mechanism through which TNS4 promotes migration and proliferation remains unclear. TNS4 belongs to the tensin family that includes focal adhesion proteins bridging the extracellular matrix with the actin cytoskeleton and involved in cellular adhesion and motility (95). However, TNS4 differs structurally from the other members (tensin1, tensin2 and tensin3) because it lacks the N-terminal region binding to the actin microfilaments. As a consequence, TNS4 cannot bind to the cytoskeleton (95, 96). Therefore, the increased migration induced by TNS4 overexpression in normal and cancer cholangiocytes is unlikely to result from a direct effect of TNS4 alone. Rather, TNS4 could displace other tensins in the focal adhesions to dissociate the structural link between the extracellular matrix and the cytoskeleton and promote motility. This switch between different tensins was shown in mammary cell lines (97). Along the same lines, how TNS4 overexpression enhances cell proliferation remains unclear. TNS4 contains a Src homology domain that allows interactions with other phosphorylated proteins and possibly connects TNS4 to intracellular signaling pathways responsible for its proliferative effects.

Transfection experiments in normal mouse cholangiocytes (NMC) and EGI-1 cells allowed to identify a transcriptional gene regulatory network, involving KRAS and SOX17, that contributes to stimulate the expression of TNS4 during cholangiocarcinogenesis. SOX17 and TNS4 colocalize in human iCCA and their levels of expression show a positive correlation in 2 distinct iCCA cohorts, which supports

the proposed link between SOX17 and TNS4. Thus, our data present SOX17 as an oncogene during cholangiocarcinogenesis, which contrasts with Merino-Azpitarte's work providing arguments supporting a tumor suppressor effect of SOX17 in CCA (hypermethylation of SOX17 promoter in CCA, reduced tumor volume and reduced cell migration of EGI-1 xenografts overexpressing SOX17) (98). Merino-Azpitarte's paper concerned full blown CCA while we studied tumor initiation in iCCA and these different stages of tumor development could contribute to the discrepancies. Earlier work in pancreatic cancer confirmed the oncogenic effect of SOX17 during the first steps of tumor development (99).

Our analyses highlighted the incomplete penetrance of the phenotype: $Kras^{G12D}$ expression combined with a DDC-diet induced iCCA in only 1 out of 3 $OpnCre^{ER}$ LSL- $Kras^{G12D}$ LSL-Rosa^{YFP} mice. The reason for this incomplete penetrance (iCCA in 1 mouse out of 3) is unclear. We can speculate that the $Kras^{G12D}$ mutation was not expressed in DDC treated $OpnCre^{ER}$ LSL- $Kras^{G12D}$ LSL-Rosa^{YFP} mice that fail to develop iCCA. Cre recombinase might have induced recombination of the YFP gene in the Rosa26 locus but not of the Kras locus. Another option is the apical extrusion process that eliminates $Kras^{G12D}$ expressing cholangiocytes. However, such apical extrusion hypothesis seems less likely because normal epithelial cells are responsible for this extrusion and our cells in principle express mutant Kras (88). Finally, our mouse colony is outbred, and incomplete penetrance may rely on genetic polymorphisms.

How DDC diet-induced cholangitis primes tumor initiation remains unanswered. Indeed, $OpnCre^{ER}$ LSL- $Kras^{G12D}$ mice devoid of DDC treatment did not show any phenotype and mice expressing wild type Kras and fed a DDC diet developed IPNB but no iCCA nor early cancer lesion. Therefore, the DDC treatment allows the oncogenic effect of the $Kras^{G12D}$ mutation. Fickert et al. described the inflammatory and cholestatic effects of DDC diets resulting from the accumulation of porphyrin plugs in the bile duct lumen that irritate the biliary epithelium and obstruct the bile flow (100). The molecular mechanisms through which inflammation triggers tumor initiation in $Kras^{G12D}$ driven cancers were studied in mouse models of pancreatic cancer (pancreatic ductal adenocarcinoma) (101). Transgenic mice expressing postnatally $Kras^{G12D}$ in acinar cells show a limited

number of low grade dysplastic lesions in the pancreas (low grade pancreatic intraepithelial neoplasia) whereas pancreatitis promotes tumor initiation in the same mice. Assi et al. showed that inflammation stimulates the expression of Kras (and Kras^{G12D}) and of several of its effectors involved in downstream signaling pathways critical for carcinogenesis such as the MAPK/ERK and the PI3K/AKT pathways (101). The same study provides evidence that this increase in expression of Kras and its effectors is controlled by the EGFR signaling. This mechanism explaining the cooperation between Kras mutation and inflammation may not be simply transposed to the liver because distinct organs present different basal level of expression of Kras (101). However, during cholangiocarcinogenesis, inflammation (and cholestasis) indirectly promote EGFR signaling through an activation of metalloproteinases able to cleave EGFR proligands to release EGF (24). p-ERK is already expressed in phenotypically normal cholangiocytes from mice expressing Kras^{G12D} even in the absence of DDC treatment. In contrast, p-AKT remains undetectable in the Kras^{G12D} expressing cholangiocytes until DDC treatment is initiated: IPNB from mice expressing wild type Kras and treated with DDC do express p-AKT. We speculate that the controlled expression of Kras effectors, if any, depends on the PI3K/AKT pathway rather than on the MAPK pathway in our iCCA model.

Alternatively, the inflammatory process induced by the DDC diet might be a source of IL-6 that activates an intracellular signaling pathway leading to STAT3 activation whose oncogenic potential was demonstrated in CCA (24). Hypothetically, the activation of the IL-6/STAT3 pathway in combination with the MAPK/ERK and/or the PI3K/AKT pathways downstream of the Kras^{G12D} mutation could favor tumor development in OpnCre^{ER} LSL-Kras^{G12D} mice fed a DDC diet whereas the activation of the MAPK/ERK and/or the PI3K/AKT pathway(s) alone is (are) not sufficient to induce carcinogenesis in the time allowed for the analysis. IL-6 and STAT3 belong to a complex positive feed-back loop described to induce cell transformation of an immortalized cell line derived from normal breast epithelial cells. bridging inflammation and cancer initiation in an artificial model (102). In that model, tumor suppressors inhibit this positive feedback loop and oncogenes activate it. Following the model, Ras is a positive regulator of this loop and in presence of oncogenic Ras mutations, the level of inflammation required to reach the threshold of activity of the positive feedback loop inducing the malignant behavior decreases. In the same lines, it is possible that in presence of

inflammation, the level of activity of the pathways downstream of Kras^{G12D} required to induce cell transformation decreases. In that hypothesis, Kras^{G12D} and inflammation cooperate because they regulate the same network.

In summary, we developed a transgenic mouse model of iCCA recapitulating faithfully the human pathology and we proposed a model of evolution from precursor lesions to cancer. However, we did not address the clinical implication of our data, namely the potential use of our data to develop screening tools for early detection of iCCA. Measuring TNS4 and SOX17 proteins in blood samples from DDC treated mice expressing wild type Kras (development of IPNB without iCCA) and in DDC treated mice expressing Kras^{G12D} mutation (development of IPNB and iCCA) would constitute a first approach to translate our fundamental findings in improved diagnosis. Still we are aware of the limitation that SOX17 is only expressed in a subset of cells, and measuring TNS4 might lack specificity since it is expressed in several tissues (prostate, breast, colon, lung and stomach) in basal conditions (95).

IV.2.2 Gallbladder cancer

IV.2.2.1 Mouse models of gallbladder carcinogenesis

The lack of genetic animal model of GBC is surprising and raises questions on potential biological properties that would be specific to gallbladder cholangiocytes. According to our spatial transcriptomic analysis of GBC and BiIN, NR2F1 is significantly downregulated in all the lesions (BiIN low grade, BiIN high grade and GBC in patient 1 and patient 2) as compared to normal epithelia. NR2F1 is a transcription factor inducing tumor cell dormancy for example in prostate cancer cells (103). We speculate that high levels of NR2F1 maintain the gallbladder cholangiocytes in a dormant state and that downregulation of this gene is required to prime tumor initiation.

IV.2.2.2 Spatial transcriptomics of human gallbladder cancer and biliary intraepithelial neoplasia

Our spatial transcriptomic analysis of GBC, BiIN low grade and BiIN high grade lesions indicates that GBC likely originates from BiIN high grade in patient 1

and from BillIN low grade in patient 2. These data challenge the proposed multistep model of carcinogenesis according to which BillIN low grade evolves to BillIN high grade and eventually to GBC (104, 105). While our work was ongoing with the analysis of our transcriptomic data, Lin et al. published in 2021 a genomic analysis of GBC and precursor lesions supporting distinct evolutionary trajectories during gallbladder carcinogenesis (67). They showed that some GBC were closely related to BillIN low grade from a genomic point of view and likely derived from BillIN low grade. Moreover, they questioned the premalignant potential of BillIN low grade and BillIN high grade lesions because some GBC displayed genomic profiles significantly different from both BillIN high and BillIN low grade. In this BillIN-independent model, GBCs are thought to derive from a putative common ancestor closer to the normal cell that could acquire full malignant potential as a result from a high mutational load (67).

Transition from precursor/premalignant lesion to cancer is essentially due to the acquisition of invasive properties, among which migration and basal membrane degradation are key elements. Acquisition of invasive properties may occur at any time during the carcinogenic process depending on the consequences of cell-intrinsic (e.g. mutational load) and cell-extrinsic (e.g. aberrant cell-cell signaling) mechanisms that can be activated stochastically. Fast and early acquisition of a high mutational load may induce malignancy *de novo*, without transit through precursor lesions that merely reflect a progressive and slower acquisition of defects. Retrospectively, it seems logical to me that the activation of such pathways occurs regardless of the histological grade of the lesion. To the best of my knowledge, the histological classification of BillIN does not fit with an accurate biological mechanism. Modifications of the size and the shape of the nuclei associated with condensation of the chromatin are the witnesses of genetic and epigenetic events affecting the ploidy of the cells, the integrity of the nuclear membrane and the location of heterochromatin. Consequently, I think that BillIN lesions should be considered as risk factors rather than premalignant lesions that necessarily evolve to malignancy. Our data suggest that inflammation primes gallbladder carcinogenesis. Gene expression profiles from patient 2, affected with primary sclerosing cholangitis, indicate that GBC likely derives from BillIN low grade in that patient. Hypothetically, a positive feed-back loop could control the acquisition of the malignant phenotype, as shown in breast epithelial cells in culture (102). In that context, it is reasonable to propose that inflammatory cytokines

activate this positive feed-back loop and by doing so decrease the level of oncogenic stress required to induce malignancy. This could explain why a GBC occurs in a BillIN low grade lesion and suggests that low grade dysplastic lesions should be taken with specific attention in patients suffering from inflammatory chronic disease.

Our spatial transcriptomic data from BillINs and GBCs analyzed in 2 patients support the BillIN dependent model proposed by Lin et al. Because their transcriptomic profiles were related, we considered a possible filiation between BillIN high grade and GBC in patient 1 and BillIN low grade and GBC in patient 2, even if this filiation cannot be proven. This prompted us to consider BillIN high grade in patient 1 and BillIN low grade in patient 2 as premalignant lesions.

Interestingly, these two premalignant lesions expressed fibrillar collagens (COL1A1) that we localized in the epithelial cells at the mRNA level . The level of expression of COL1A1 was higher in lesions considered as premalignant. In an in vitro model of cancer invasion, Glentis and coworkers showed that large bundles of extracellular matrix produced by CAF along the basement membrane contributed to destroy it and to favor invasion.(72). My hypothesis is that the fibrillar collagen produced and likely secreted by the epithelial cells in our premalignant lesions could also modify the properties of the basement membrane, weaken it and favor transition from an in situ process to an invasive cancer. (Figure 1).

Another option that remains purely speculative is that a shift in the program of extracellular matrix production occurs during carcinogenesis. In other words, normal epithelial cells contribute to the production of their basement membrane essentially made of collagen 4, collagen 6 and laminin. It is possible that premalignant cells stop the production of this normal extracellular matrix to produce a new one, made of fibrillar collagen that promotes migration and invasion. (Figure 1).

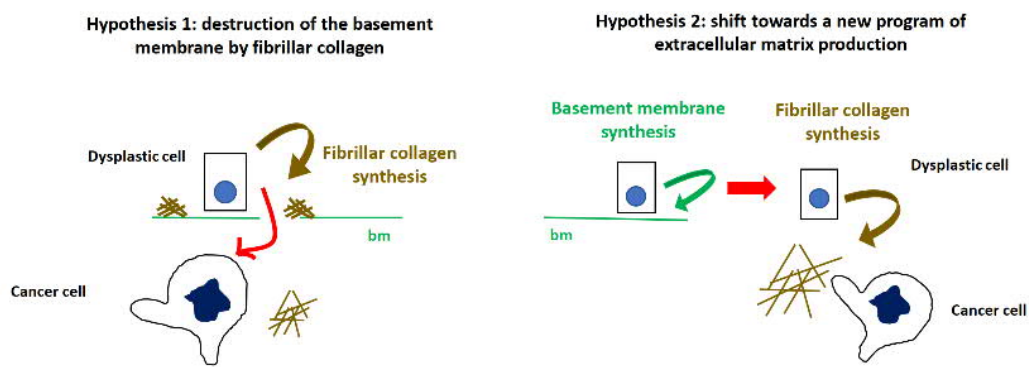


Figure 1: Transition from premalignant lesions to invasive cancer: hypotheses to explain the role of the fibrillar collagen detected in epithelial cells in LG BilIN in patient 2 and in HG BilIN in patient 1. Bm= basement membrane

I then classified the BilIN low grade from patient 1 and BilIN high grade from patient 2 as “low risk lesions”. In contrast, GBC from both patients, BilIN high grade in patient 1 and BilIN low grade in patient 2 were categorized as “high risk lesions”, i.e. cancers and premalignant lesions. I looked for common transcriptomic events occurring in “high risk lesions” as compared to “low risk lesions” to investigate the carcinogenesis regardless of the histological grade of BilIN.

An interesting perspective is the study of the ZNF100 transcription factor whose activity levels fit with the sequence of tumor progression suggested by our transcriptomic data. This observation emerged from a bioinformatic analysis using the SCENIC (single cell regulatory network inference and clustering) algorithm (106). The latter defines regulons, i.e. modules of co-expressed/co-occurrent genes

presenting an enrichment in their promoters for a common transcription factor binding site. The activity of the regulons can be measured in each region of interest and the level of activity of one single regulon between different regions of interest can be compared. Following this analysis, ZNF100 regulon activity decreases in “high risk lesions” in both patients as compared to “low risk lesions”. Little is known about this zinc finger transcription factor but the literature indicates a potential tumor suppressor role in colorectal cancer because ZNF100 belongs to the most hypermethylated genes in colorectal cancer (107). Therefore, the decrease of activity of ZNF100 regulon could drive gallbladder carcinogenesis.

Finally, SEMA4A was significantly downregulated in GBC and premalignant lesions (BillN high grade from patient 1 and BillN low grade from patient 2) from both patients as compared to normal epithelia. In contrast, BillN high grade from patient 2, which at the transcriptomic level is far from GBC, displayed levels of expression of SEMA4A comparable to those of normal epithelium. SEMA4A belongs to the family of semaphorins which are membrane-bound or secreted proteins usually acting as signaling ligands to modulate the motility and the morphology of the cells. The main target of semaphorin-induced signaling is thus the cytoskeleton (108). Shortly after their description as axon guidance molecules, the role of semaphorins in cancer began to be elucidated (109). SEMA3B and SEMA3F were identified in the 3p21.3 region of chromosome 3 which is frequently lost in lung and breast cancers (109). SEMA3F and SEMA3B are also downregulated in squamous cell carcinoma (109) and their tumor suppressor role was shown in lung cancer cell lines (110). Incubation of breast cancer cells with recombinant SEMA3A, another member of the family, impeded their migration (111). Several lines of evidence thus support a tumor suppressor role for semaphorins in cancer. Importantly, their effect are cell specific and the role of SEMA4A signaling in gallbladder epithelium is unknown. Still, germline loss-of-function mutations in SEMA4A were found in patients affected with familial colorectal cancer type X (112) which suggest a tumor suppressor effect of SEMA4A in that specific cancer. Although a subsequent study did not find this variant in another colo-rectal cancer cohort, I considered that the downregulation of SEMA4A detected in our transcriptomic analysis could drive gallbladder carcinogenesis.

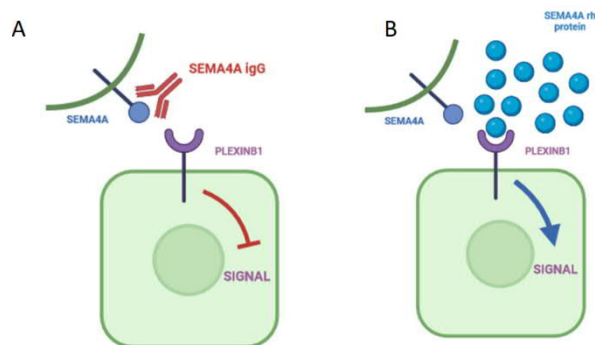
We showed tumor suppressive properties of SEMA4A using non-tumoral gallbladder organoids and EGI-1 xenografts treated with SEMA4A IgG antibody or

EGI-1 cells in culture incubated with a soluble form of SEMA4A recombinant protein. SEMA4A IgG antibody targets the extracellular part of the SEMA4A and prevents SEMA4A from binding to its main receptor, Plexin B1 (Figure 2A). This SEMA4A IgG antibody thus decreases SEMA4A-induced signaling and functions as a blocking antibody (113).

Recombinant human SEMA4A protein corresponds to the extracellular domains of the protein that can be cleaved and released physiologically. SEMA4A recombinant human protein can thus bind to the main receptor of SEMA4A, Plexin B1 and activate SEMA4A induced signaling.

Inhibition of SEMA4A signaling promoted BillN development in non-tumoral gallbladder organoids and accelerated tumor growth of EGI-1 xenografts. At the opposite, activation of SEMA4A-induced signaling reduced clonogenicity and migration of EGI-1 cells in culture and microvascular invasions in EGI-1 xenografts.

Figure 2: Mode of action of SEMA4A IgG antibody (A) and SEMA4A recombinant human protein (B).



How SEMA4A and Plexin B1 interact remains unanswered. We can propose three hypotheses (Figure 3).

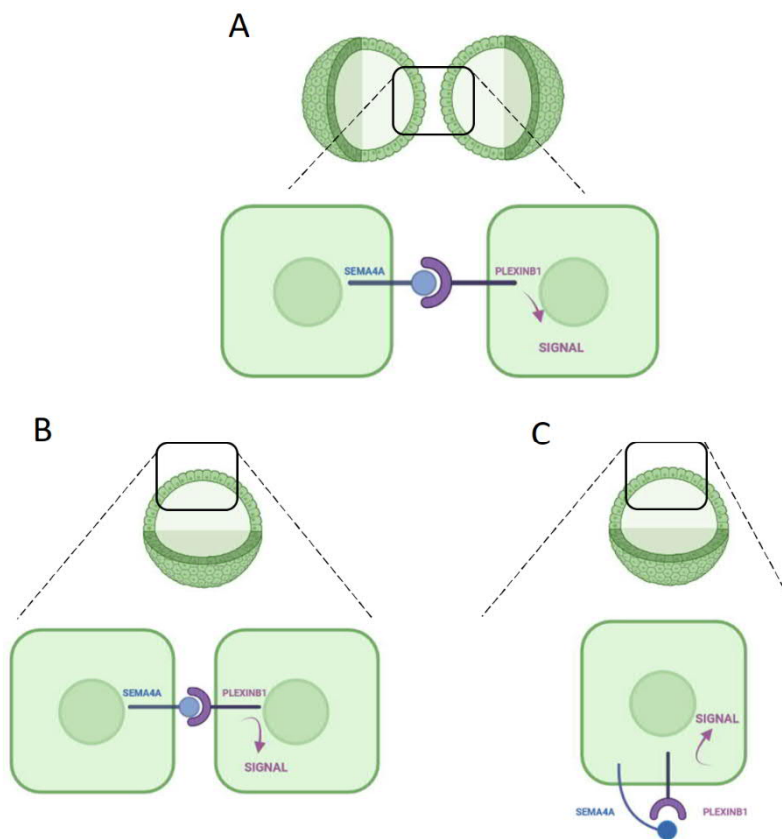


Figure 3: Possible interactions between SEMA4A and PlexinB1.

A first hypothesis (Figure 3A) consists of an interaction between two different organoids. In that option, SEMA4A expressed by an epithelial cell from an organoid would bind to PlexinB1 located on an epithelial cell of another organoid. This seems rather unlikely because we did not observe contacts between cultured organoids covering extended areas of organoid epithelium. Another option is that this interaction occurs within a single organoid (Figures 3B and 3C). Two different

epithelial cells from the same organoid could express SEMA4A and Plexin B1 respectively, inducing a paracrine signal in the epithelial cell bearing PlexinB1. Finally, the same cell could express both SEMA4A and PlexinB1 (Figure 3C).

These hypotheses remain purely speculative since the signaling pathway mediating the effects of SEMA4A in the gallbladder has not been investigated. Still, given the possible cleavage of the extracellular domain of SEMA4A in physiological conditions (114), SEMA4A can be detected in the serum of patients (115). A decrease in blood levels of SEMA4A may potentially be used as a screening tool to detect GBC at an early stage. However, further investigations are needed to check the reproducibility of these observations in larger cohorts and to check the bioavailability of SEMA4A.

To conclude, the present thesis focus on transcriptomic events driving epithelial transitions during carcinogenesis. Precursor lesions of BTC have been poorly studied until now. They could develop from several cell types and the histogenic sequence from low grade to high grade dysplastic stages and eventually carcinoma is likely not applicable in all cases. Several questions remain unanswered. How do these precursor lesions interact with the immune microenvironment? Can precursor lesions be a target of anti-tumor immunity and inhibit evolution of the lesions towards malignancy ? Precursor lesions contain several subclones and malignant transformation requires selection of subclones with proliferative advantage (116). How does this selection occur? Are there cross-talks between different cell populations within the precursor lesions, with -for instance- one population inducing the senescence of another? Does the dynamic evolution of these lesions depend on the micro-environment ? Answering these questions is the obvious goal to reach to acquire a full understanding of tumor pathogenesis.

V. References

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VI. Annex

VI.1 Annex I : review

REVIEW



Genetically engineered animal models of biliary tract cancers

Sophie Pirene and Frédéric P. Lemaigre

Purpose of review

Biliary tract cancers which include intrahepatic and extrahepatic cholangiocarcinomas and gallbladder cancer, are characterized by poor outcome. Therefore, identifying the molecular mechanisms of the disease has become a priority. However, such identification has to cope with extreme heterogeneity of the disease, which results from the variable anatomical location, the numerous cell types of origin and the high number of known genetic alterations.

Recent findings

Animal models can develop invasive and metastatic tumours that recapitulate as faithfully as possible the molecular features of the human tumours. To generate animal models of cholangiocarcinoma, investigators resorted to the administration of carcinogens, induction of cholestasis, grafting of tumour cells and induction of genetic modifications.

Summary

Here, we summarize the currently available genetically engineered animal models, and focus on mice and zebrafish. The experimental strategies that were selected to induce cholangiocarcinoma in a time-controlled and cell-type-specific manner are critically examined. We discuss their strengths and limitations while considering their relevance to human pathophysiology.

Keywords

biliary intraepithelial neoplasia, cholangiocarcinoma, intraductal papillary neoplasm of bile ducts, transgenic mouse, transgenic zebrafish

INTRODUCTION

Biliary tract cancers are rare tumours with dismal prognosis. They constitute a group of heterogeneous diseases the incidence rate of which is rising while exhibiting significant geographical variations [1[•]]. Growing attention to the disease in recent years revealed a global increase in risk factors, but also a number of epidemiological biases resulting from nonstandardized nomenclature, and from misclassification of cholangiocarcinomas [1[•],2]. Therefore, when considering the mechanisms driving initiation and development of cholangiocarcinoma one must examine with care the type or subtype of studied cholangiocarcinoma, and the cellular and molecular specificities at the origin of the observed heterogeneity. Similarly, animal models that recapitulate initiation and progression toward invasive and metastatic cholangiocarcinoma should reflect the histomorphological and molecular features of the human tumour subtypes. Other reviews discussed in depth how animal models contribute to our understanding of cholangiocarcinoma development and to the design of therapy [3–6]. Here, we

focus on genetically engineered animal models and discuss their strengths and weaknesses.

CHOLANGIOCARCINOMA: MALIGNANT AND PREMALIGNANT TUMOURS

The most widely used cholangiocarcinoma classification is based on the anatomical topography of tumours, and distinguishes between intrahepatic and extrahepatic carcinomas [7[•],8[•],9]. Intrahepatic cholangiocarcinomas are peripheral to the second order branches of the bile ducts; they represent approximately 10% of all cholangiocarcinomas. Extrahepatic cholangiocarcinomas are subdivided into perihilar and distal tumours, the former

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KEY POINTS

- Biliary tract cancers are rare tumours with dismal prognosis, and a subset of intrahepatic cholangiocarcinoma display rising incidence. Recent work has highlighted extreme heterogeneity of the disease which is manifested by the variable intrahepatic and extrahepatic location, a high number of driver mutations, the heterogeneous histopathology, and multiple cell types of origin.
- Mutations occurring in human biliary tract cancer can be reproduced in genetically engineered mice and zebrafish. These animal models recapitulate several features of tumour development. However, attention should be paid to cell-type specificity, timing of genetic recombination and oncogenic gene dosage while interpreting the results.
- Characterization and classification of biliary tract cancers are making progress in humans. We anticipate that future analyses and generation of animal models will take these evolutions into consideration, while focusing on mechanisms that control stepwise tumorigenesis.

emerging from the right, left and common hepatic ducts, the latter developing from the common bile duct downstream of the origin of the cystic duct. Although gallbladder carcinoma displays molecular and clinical specificities, it is usually considered together with extrahepatic duct carcinoma.

Complementary to the anatomical classification, histological analyses showed that extrahepatic cholangiocarcinoma are predominantly mucin-producing adenocarcinoma. In contrast, intrahepatic cholangiocarcinoma exhibit more variability. Two subtypes were identified. Small duct subtype preferentially occurs at the periphery of the liver lobes and is characterized by tubular-type adenocarcinoma with low columnar to cuboidal cells and possible ductular component without mucin production. The large duct subtype is mainly found at the proximity of the hilum and typically displays a tubular pattern with mucin-producing and columnar cells. Rare histological variants include combined hepatocellular-cholangiocarcinomas which associate features of cholangiocarcinoma and hepatocellular carcinoma [7[•],8[•],10]. Meanwhile, expert pathologists proposed to classify the tumours on the basis of histological criteria that were supposed to reflect the cell type of origin [11,12]. Indeed, cholangiocytes are heterogeneous at the histological and transcriptomic level, suggesting that distinct types of cholangiocytes might be at the origin of various histomorphological forms of cholangiocarcinoma [11,12,13^{••},14^{••}]. Moreover, biliary tract cancers

can potentially arise not only from cholangiocytes, but also from peribiliary glands, progenitor cells and hepatocytes [15^{••},16–21], thereby increasing the potential for heterogeneity in tumour phenotype and adding to the complexity of cholangiocarcinoma tumorigenesis.

The study of cholangiocarcinoma tumorigenesis enabled investigators to identify biliary intraepithelial neoplasia (BillIN) and intraductal papillary neoplasms of bile ducts (IPNB) as noninvasive precursor lesions [22,23]. Significantly, these neoplasia bear similarities with precursors lesions of pancreatic cancer [24[•]]. BillINs are microscopic lesions presenting as flat or micropapillary dysplasia of the biliary epithelium, and develop in large bile ducts and gallbladder. IPNBs are macroscopic lesions of the large ducts characterized by papillary structures with fibrovascular core, and are detected intrahepatically and extrahepatically. There are also some histological arguments that von Meyenburg complexes, also called microhamartomas, may in rare instances evolve toward invasive cholangiocarcinoma [25]. They present as irregularly shaped and dilated ductal structures embedded in fibrous stroma.

Intraductal tubulopapillary neoplasm and mucinous cystic neoplasms are also considered premalignant lesions, but are more rare than BillINs, IPNBs or von Meyenburg complexes. Intraductal tubulopapillary neoplasm contain densely packed tubular glands with little mucin [23]. Mucinous cystic neoplasms form large unilocular, multilocular or multicystic structures, not connected to the biliary tree, with variably mucin-producing epithelium and ovarian-type stroma; they display low or intermediate dysplasia, but can evolve to carcinoma *in situ* [23,26].

GENETIC ANOMALIES IN BILIARY TRACT CANCER AND PREMALIGNANT LESIONS

Genetic, biochemical and cell biology approaches identified several signalling pathways driving tumour progression. Given that chronic inflammation is a major risk factor for cholangiocarcinoma, it is not surprising that pathways typically associated with an inflammatory environment were found to initiate and promote biliary tract cancer. Moreover, pathways regulating embryonic development and regeneration of the bile ducts are often associated with tumour progression. Description of these tumour-promoting pathways is beyond the scope of the present article and we refer to recent reviews for an in-depth discussion [27–29].

The mutational landscape of intrahepatic and extrahepatic cholangiocarcinoma has been described in high-throughput studies and discussed in

excellent reviews [8[•],30–41]. Mutations include copy number alterations, gene fusions, noncoding promoter mutations, alterations of the 3'untranslated regions and coding mutations. A large number of genes were found to be mutated in cholangiocarcinoma and investigators attempted to classify tumours on the basis of signalling modules that combine sets of gene mutations involving *RAS*, *TGFβ-SWI/SNF-MYC*, *RB* or epigenetic control genes [38]. Following parallel approaches, others clustered tumour types by associating genomic or epigenomic alterations with clinical features [40,42,43]. From these analyses, it is concluded that the frequency of a specific gene alteration may vary according to the anatomical site, but that tumours within a same location can display markedly different mutational spectra. Moreover, tumours located in distinct areas of the biliary tree sometimes show overlapping features, and intrahepatic cholangiocarcinoma can also display molecular features overlapping with hepatocellular carcinoma [42].

Interpretation of genetic analyses is further complicated by the fact that the mutational spectrum of a tumour evolves during the tumorigenic processes and influences the tumour phenotype. To illustrate this notion, hepatocellular carcinoma can evolve toward combined hepatocellular-cholangiocarcinoma, as evidenced in a mouse model where *Phosphatase and tensin homolog (Pten)* and *Tp53* are codeleted in early postnatal hepatic progenitor cells expressing *Prominin1 (Prominin-CreER^{T2}/Pten^{loxP/loxP}/Tp53^{loxP/loxP})*. These mice develop hepatocellular carcinoma. However, when their tumour cells were grown as organoids and subsequently implanted in mice, some organoid-derived tumours developed as mixed hepatocellular-cholangiocarcinoma, thereby providing evidence that cholangiocarcinoma phenotypes evolve as a function of time [44[•],45]. In that respect, the field of liver cancer would certainly benefit from implementing tumour phylogeny analyses [46].

A way to characterize the role of genetic alterations in progression from premalignancy toward malignancy and metastasis consists in searching for mutations already present in precursor lesions [47–51]. Approximately one third of intrahepatic BilINs display activating *KRAS* mutations, and progression toward advanced stages of BilIN is associated with decreased *SMAD4* expression. In IPNBs, activated *KRAS* and loss of *TP53* and *p16* function are detected at early stages of adenoma-to-carcinoma transition. Significantly, *KRAS* mutations are absent from oncogenic type IPNBs. Progressive downregulation of *SMAD4* is mainly observed in high grade IPNB. Guanine nucleotide-binding protein g(s) alpha subunit (*GNAS*) mutations are variably detected in IPNBs depending on patient cohorts; β-catenin

(*CTNNB1*), ErbB2 Receptor Tyrosine Kinase 2 (*ERBB2*; also called *HER2*) and epidermal growth factor receptor mutants are rarely found.

GENETICALLY-ENGINEERED ANIMAL MODELS OF BILIARY TRACT CANCERS

Various animal models of cholangiocarcinoma were designed based on administration of carcinogens, induction of cholestasis, grafting of tumour cells and induction of genetic modifications [5,6]. Here, we discuss genetically-modified animal models and focus on mice (*Mus musculus*) and zebrafish (*Danio rerio*) which are the most popular in cancer research. A list of genetically engineered mouse models developing cholangiocarcinoma is provided in Table 1 [15^{••},17–21,52–64,65^{••}]. The Table lists the various transgenic approaches that were implemented and highlights a number of criteria that one needs to keep in mind when considering the relevance and ease of use of the mouse models.

As stated above, cholangiocarcinoma may develop from several cell types, indicating that careful assessment of the cell type which expresses the transgene is mandatory. Determining the cell type of origin of liver regeneration by means of transgene-dependent lineage tracings revealed many pitfalls when selecting a transgene supposedly active in a cell-specific way [66]. In many instances, it appeared that cell-type specificity was broader than anticipated and confounded interpretation of the results. Cre-mediated induction of gene recombinations for generating cholangiocarcinoma faces the same issue. Cre-driver genes such as *Alb-Cre*, or *Alfp-Cre* are expected to induce recombination at loxP-flanked genes in prenatal liver, and more specifically in hepatoblasts which are the precursors of adult hepatocytes and cholangiocytes. The efficiency of Cre-mediated recombination, that is the percentage of cells in which the loxP-flanked target alleles have been recombined, depends more often on the structure of the target gene than on the expression level of the Cre-driver gene. Moreover, recombination might occur early or late during liver development, again depending on the target gene. In that context, Cre-mediated induction of gene mutations at the embryonic stage is considered to recapitulate a progenitor cell-initiated cholangiocarcinoma. However, one must bear in mind that hepatoblast differentiation is highly dynamic and that the cellular context of a hepatoblast at an early stage of liver development differs from that in hepatoblasts at the end of gestation. Early postnatal hepatic cells are not fully mature, and this likely explains why *Alb-CreER/Pten^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}* mice injected with tamoxifen at postnatal day 10 develop

Biliary tract

Table 1. Genetically engineered mouse models of cholangiocarcinoma

Genotype	Targeted cell type	Onset of transgene activity	Lesion	Latency period for CCA	Reference
<i>Alb-Cre/Smad4^{loxP/loxP}/Pten^{loxP/loxP}</i>	Hepatoblasts, hepatocytes, cholangiocytes, activated stellate cells	Prior birth; validated by recombination of <i>Rosa26R^{lacZ}</i> at P15	Papillary dysplasia, iCCA, salivary gland tumor (rarely)	4–7 Months	[52]
<i>Alb-Cre/Pten^{loxP/loxP}</i>			iCCA, HCC, salivary gland Tumor (rarely)	1 year	
<i>Alb-Cre/Tp53^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}</i>	Hepatoblasts, hepatocytes, cholangiocytes, activated stellate cells	Prior birth	iCCA, HCC, mixed HCC-CCA, IPNB, VMC	13–32 Weeks	[53,65 [■]]
<i>Alb-Cre/Tp53^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}+DDC</i>			iCCA, mixed HCC-CCA, no HCC		
<i>Alb-Cre/Tp53^{loxP/+}/loxP-stop-loxP-Kras^{G12D}</i>			iCCA, HCC, mixed HCC-CCA	32–62 Weeks	
<i>Alb-Cre/Tp53^{+/-}/loxP-stop-loxP-Kras^{G12D}</i>			iCCA (rarely)	36 Weeks	
<i>Alb-Cre/Pten^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}</i> <i>Alb-Cre/Pten^{loxP/+}/loxP-stop-loxP-Kras^{G12D}</i> <i>Alb-Cre/Pten^{+/-}/loxP-stop-loxP-Kras^{G12D}</i> <i>Alb-Cre/Pten^{G12D}/loxP-stop-loxP-Kras^{G12D}</i> <i>Alb-CreERT2/Pten^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}</i>	Hepatoblasts, hepatocytes, cholangiocytes, Adult: hepatocytes, cholangiocytes (rarely) Newborn: hepatocytes, cholangiocytes	Prior birth Starting from tamoxifen injection	iCCA, papillary premalignant tumours iCCA (unfrequent), hepatocellular dysplasia Hepatocellular dysplasia Tamoxifen administered at 8 weeks: HCC Tamoxifen administered at P10: HCC, iCCA	7 Weeks 6 Months 2 Months	[54,55]
<i>Alb-CreERT2/loxP-stop-loxP-Notch intracellular domain+thioacetamide</i>	Hepatocytes	Starting from tamoxifen injection	iCCA	14 Weeks	[17]
<i>K19-CreERT2/loxP-stop-loxP-Kras^{G12D}/Pten^{loxP/loxP}</i>	Cholangiocytes, adult progenitors, peribiliary glands	Starting from tamoxifen injection	Premalignant iCCA, papillary dysplasia in GB and EHBD; multiorgan defects	1 Month	[54]
<i>Sox9-CreERT2/loxP-stop-loxP-Kras^{G12D}/Pten^{loxP/loxP}</i>	Cholangiocytes, periportal hepatocytes	Starting from tamoxifen injection	hyperplasia in IHBD, EHBD and GB; biliary adenoma of IHBD	3 Months	[55]
<i>Sox9-CreERT2/loxP-stop-loxP-Kras^{G12D}/Tp53^{loxP/loxP}</i>	Cholangiocytes, periportal hepatocytes	Starting from tamoxifen injection	BillIN, iCCA, HCC (rarely)	30 Weeks	[65 [■]]
<i>K19-CreERT2/loxP-stop-loxP-Kras^{G12D}/Tbr1^{loxP/loxP}/Cdh1^{loxP/loxP}</i>	Cholangiocytes, adult progenitors, peribiliary glands	Starting from tamoxifen injection	eCCA	1 Month	[15 [■] ,56]
<i>K19-CreERT2/loxP-stop-loxP-Kras^{G12D}/Tbr1^{loxP/loxP}</i> <i>Alb-Cre/loxP-stop-loxP-Kras^{G12D}/Tbr1^{loxP/loxP}/Cdh1^{loxP/loxP}</i>			Hyperplasia in EHBD		
<i>Alb-Cre/loxP-stop-loxP-Kras^{G12D}/Cdh1^{loxP/loxP}</i>	Hepatoblasts, hepatocytes, cholangiocytes, activated stellate cells	Prior birth	Hilar CCA, dysplasia of peripheral IHBD HCC, iCCA, mixed HCC-iCCA	2 Months	
<i>K19-CreERT2/loxP-stop-loxP-Kras^{G12D}</i>	Cholangiocytes, adult progenitors, peribiliary glands	Starting from tamoxifen injection	No biliary tumor		
<i>K19-CreERT2/Tbr1^{loxP/loxP}</i> <i>K19-CreERT2/Cdh1^{loxP/loxP}</i>			No biliary tumor		
<i>Alb-Cre/loxP-stop-loxP-Idh2^{R172K}/loxP-stop-loxP-Kras^{G12D}</i>	Hepatoblasts, hepatocytes, cholangiocytes, activated stellate cells	Prior birth	iCCA	47 Weeks	[21]
<i>Alb-Cre/loxP-stop-loxP-Kras^{G12D}</i>			HCC (rarely)	70 Weeks	
<i>Alfp-Cre/loxP-stop-loxP-Notch1 intracellular domain</i>	Hepatoblasts	Prior birth	iCCA after transplantation of Notch1 overexpressing cells in immunodeficient mice		[57]

Table 1 (Continued)

Genotype	Targeted cell type	Onset of transgene activity	Lesion	Latency period for CCA	Reference
<i>Ah-Cre/Pten^{loxP/loxP}</i>	Hepatocytes, cholangiocytes	Starting from tamoxifen and β -naphthoflavone injection	iCCA, HCC, mixed HCC–CCA, VMC, hyperplasia in IHBD, EHBD, GB; multiorgan defects		[58]
<i>Ah-Cre/loxP-stop-loxP-Kras^{G12V}/Pten^{loxP/loxP}</i>			GB adenocarcinoma, noninvasive papillary hyperplasia in GB and IHBD, multiorgan defects		
<i>Ah-Cre/loxP-stop-loxP-Kras^{G12V}</i>			no lesion		
<i>Alfp-Cre/p53^{loxP/loxP}</i>	Hepatoblasts	Prior birth	Mixed HCC-CCA, HCC	14–20 Months	[59]
<i>Alb-Cre/Hspd1^{loxP/loxP}</i>	Hepatoblasts, hepatocytes, cholangiocytes, activated stellate cells	Prior birth	BillIN, iCCA after transplantation of <i>Hspd1</i> –/– cells in immunodeficient mice		[60]
<i>Alb-Cre/Nf2^{loxP/loxP}</i>	Hepatoblasts, hepatocytes, cholangiocytes, activated stellate cells	Prior birth	HCC, cholangiolocellular iCCA	30 Weeks	[20]
<i>BKS-ErbB2A</i>	Hepatoblasts	Prior birth	GB adenocarcinoma iCCA cystic duct adenocarcinoma Common bile duct adenocarcinoma	0.5 Months 3 Months 0.5 Months 1 Month	[61]
<i>Pdx1-Cre/loxP-stop-loxP-Kras^{G12D}</i>	Extrahepatic and GB cholangiocytes, pancreas	Prior birth	GB adenoma		[62]
<i>Lxrb</i> –/– females			GB adenoma, GB adenocarcinoma	19 Months	[70]
<i>Prominin-CreERT2/Pten^{flax/flax}/Tp53^{flax/flax}</i>	Postnatal progenitor cells	Starting from tamoxifen injection	HCC, iCCA after transplantation		[44 [■]]
Transposase-mediated expression of Notch intracellular domain	Hepatocytes	Starting from transposase-mediated integration in genome	iCCA	20 Weeks	[18]
Transposase-mediated expression of Notch intracellular domain + Akt	Hepatocytes	Starting from transposase-mediated integration in genome	iCCA	4.5 Weeks	
Transposase-mediated expression of Yap + p53 shRNA + Notch intracellular domain	Hepatocytes	Starting from transposase-mediated integration in genome	iCCA	6 Weeks	[19]
Transposase-mediated expression of active Akt + β -catenin; inflammatory stimulus (DDC, CCl4)	Hepatocytes	Starting from transposase-mediated integration in genome	Hepatocellular adenoma, mixed HCC–CCA, hepatoblastoma (unfrequent)	11–17 Weeks	[63]
Transposase-mediated expression of active Akt + N-Ras ^{G12V} ; inflammatory stimulus (DDC)	Hepatocytes	Starting from transposase-mediated integration in genome	HCC, iCCA, mixed HCC–iCCA	8–10 Weeks	
Transposase-mediated expression of active Akt + YAP; inflammatory stimulus (IL33)	Cholangiocytes	Starting from transposase-mediated integration in genome	iCCA	10 Weeks	[64]
<i>AAV8-TBG-Cre/Tp53^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}</i>			HCC (rarely)		[65 [■]]
<i>AAV8-TBG-Cre/Tp53^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}</i> + inflammatory stimulus (DDC)			iCCA, HCC, mixed HCC–iCCA	2 Months	

List of genetically engineered mouse models developing cholangiocarcinoma. AAV, adeno-associated virus; BillIN, biliary intraepithelial neoplasia; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; eCCA, extrahepatic cholangiocarcinoma; EHBD, extrahepatic bile duct; GB, gallbladder; HCC, hepatocellular carcinoma; iCCA, intrahepatic cholangiocarcinoma; IHBD, intrahepatic bile duct; IPNB, intraductal papillary neoplasm of bile ducts; VMC, Von Meyenburg complex.

hepatocellular carcinoma and cholangiocarcinoma, while the same mice injected at 8 weeks of age only develop hepatocellular carcinoma [54]. Importantly, hepatoblasts do not necessarily reflect the cellular state of adult progenitors, and there is evidence in adult liver for the existence of several types of progenitors. Embryonic progenitors are hypersensitive to oncogenic stress, and tumorigenesis proceeding rapidly after birth may reflect such hypersensitivity. This is the case for *Bk5-ErbB2* mice which overexpress *ErbB2* in prenatal liver and develop noninvasive papillary lesions and a few invasive glands suggestive of gallbladder carcinoma as early as 2 weeks after birth [61]. Therefore, caution is required when interpreting the molecular processes occurring in the mice, since gallbladder cancer in humans does normally not develop in childhood or in sexually immature individuals.

The issue of cell-type specific gene modification is well recognized by scientists in the study of cholangiocarcinoma. Still, the lack of appropriate biological tools may hamper accurate investigation of tumorigenic processes. Nakagawa *et al.* collected an impressive body of evidence supporting that peribiliary glands are at the origin of extrahepatic cholangiocarcinoma. They used a *tour-de-force* mouse model that combined activation of *Kras* with inactivation of *TGF β receptor type II* (*TbrII*) and *E-cadherin* (*Cdh1*) under control of *Krt19-CreER^{T2}* (also called *K19-CreER* or *CK19-CreER*), a CreER gene driver that is active in intrahepatic, extrahepatic and gallbladder cholangiocytes [15⁶⁶,67]. Unfortunately, formal proof that peribiliary glands were at the origin of the tumours could not be provided due to the lack of peribiliary gland-specific Cre-recombinase.

In those cases where cell-type specificity could be well controlled with help of appropriate genetic engineering tools, important conclusions were drawn about the cell type of origin of tumours. The high hepatocyte-specificity of hydrodynamic tail vein injection of plasmids enabled Sleeping Beauty transposase-mediated genome integration of DNA coding for Notch and Akt, eventually resulting in cholangiocarcinoma. This experiment, as well as those from others, allowed to demonstrate unambiguously that cholangiocarcinoma can develop from hepatocytes [17,18]. Whether it does so in humans will prove hard to demonstrate. Along the same lines, *Krt19-CreER^{T2}* has a limited efficiency of 32% in intrahepatic cholangiocytes but is highly specific for this cell type, allowing to demonstrate that cholangiocytes are the cell type of origin in at least a subset of cholangiocarcinomas [53,67].

Temporal control of transgene recombination by using tamoxifen-inducible CreER recombinase

circumvents in part the timing uncertainties associated with noninducible Cre drivers. However, administration of tamoxifen impacts liver cell homeostasis and can delay tumour development [68]. Injection of tamoxifen is sufficient to induce *Sox9* expression in periportal hepatocytes and to activate *Sox9-CreER* in those cells which otherwise express little or no detectable *Sox9* [69]. This may contribute to the diversity of liver tumours that arise in *Sox9-CreER^{T2}/loxP-stop-loxP-Kras^{G12D}/Tp53^{loxP/loxP}* mice [65⁶⁸]. Indeed, in addition to cholangiocarcinoma, likely derived from gene recombination in *Sox9*-positive cholangiocytes, these mice develop a small number of hepatocellular carcinoma potentially derived from periportal hepatocytes. Tamoxifen is a mixed agonist/antagonist of the estrogen receptor, and since some tumours are estrogen-dependent, as illustrated by *Lxrb^{-/-}* mice which only develop estrogen-dependent gallbladder carcinoma in females [70], the long-term impact of short-term tamoxifen administration should not be underestimated. Alternatives to tamoxifen exist such as tetracyclin-controlled gene expression. This was implemented in zebrafish and mice. Overexpression of the proteasome protein Gankyrin as a Gankyrin-Hcred fusion protein in zebrafish liver under control of a hepatocyte-specific *fabp10a* promoter-inducible system lead to intrahepatic cholangiocarcinoma [71]. Expression of the hepatitis B virus X protein and hepatitis C virus core protein under control of a tetracycline-repressible activator driven by the hepatocyte-specific *L-Fabp* promoter lead to fibrosis and eventually cholangiocarcinoma [72]. In mice, tetracycline-induced expression of Isocitrate dehydrogenase (*Idh*) mutant demonstrated that it contributes to dedifferentiation of hepatocytes which subsequently can evolve to cholangiocarcinoma when combined with *Kras* activation [21].

Noncell autonomous effects can also be investigated accurately using biological tools that display well controlled cell-type specificity. Using mutant zebrafish, Yan *et al.* mimicked non-alcoholic steatohepatitis-induced TGF β 1 overexpression and showed that cholangiocarcinoma can develop from cholangiocytes exposed to chronic overexpression of *Tgfb1a* in adjacent hepatocytes. Overexpression of *Tgfb1a* concomitantly induces hepatocellular carcinoma and cholangiocarcinoma, but the requirement of activated pathways differed in the two tumour types. Consistent with the analysis of human diseased livers, cholangiocarcinoma in zebrafish required both Smad and Erk-mediated *Tgfb1a* signalling, whereas Smad was dispensable and Erk required in hepatocellular carcinoma [73].

Oncogenic dosage and mutant copy number influence cancer progression [74], and transgenic animal models enabled investigators to address their role in cholangiocarcinoma progression. This is well illustrated when comparing *Alb-Cre/Tp53^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}* and *Alb-Cre/Tp53^{loxP/+}/loxP-stop-loxP-Kras^{G12D}* mice which differ only by one mutant *Tp53* allele. The two mouse models developed similar lesions but cholangiocarcinoma occurred much later when only one *Tp53* allele was inactivated [53]. Dosage can also dictate the fate of cells undergoing carcinogenesis, as suggested by the observation that homozygous inactivation of *Pten* in *Alb-Cre/Pten^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}* mice only develop cholangiocarcinoma, whereas heterozygous *Pten* mutation in *Alb-Cre/Pten^{loxP/+}/loxP-stop-loxP-Kras^{G12D}* mice developed tumours with both hepatocyte and cholangiocyte differentiation [54]. Similar observations were made in zebrafish. Wang *et al.* [75] expressed mutant *Nras^{Q61K}* fused to *eGFP* in hepatocytes under control of the *fabp10* promoter and this led to development of hepatocyte-derived cholangiocarcinoma. Significantly, overexpression of another Ras mutant, namely *Kras^{G12V}*, under control of the same *fabp10* promoter did not induce cholangiocarcinoma [76], but hepatocellular carcinoma, which the authors interpret as resulting from different levels of expression of the Ras proteins.

Although animal models are ideal to investigate tumour progression from premalignant to malignant states, less attention has been paid to the mechanisms promoting such transitions. Several mouse models of cholangiocarcinoma mention the occurrence of biliary hyperplasia (Table 1). Human premalignant lesions such as BillN were explicitly referred to in some cases, and we point out the work of Hill *et al.* [65^{***}] who demonstrated that BillNs only form when tumorigenesis develops from cholangiocytes, not from hepatocytes. Cholangiocarcinomas developing in the neighbourhood of IPNB or von Meyenburg complexes were found in *Alb-Cre/Tp53^{loxP/loxP}/loxP-stop-loxP-Kras^{G12D}* mice [53], yet without molecular evidence that the IPNBs or von Meyenburg complexes were at the origin of the cancer.

Finally, investigators who generated animal models of cholangiocarcinoma paid attention to the clinical relevance of the tumours by highlighting the histological similarities between human and animal lesions. Published data do not easily enable to determine whether one animal model is histologically more relevant than another, in particular in a context where histopathological classification of human cholangiocarcinoma is evolving, and where animal models unveil early-stage lesions that are

uncommonly detected in humans. The most recent WHO classification of human lesions [7[¶]] might serve as an important reference in future analyses of animal models.

CONCLUSION

Genetically engineered animal models have their strengths and weaknesses. They remain invaluable for the study of the disease as they offer the possibility to combine recapitulation of genetic or epigenetic defects, associated or not with risk factors, to induce progression from normal to premalignant, invasive and metastatic state, *in vivo* and *in situ*. Future work will certainly pay more attention to molecular mechanisms driving such transitions, while maintaining the highest possible relevance to human cholangiocarcinoma. The ever growing complexity of cholangiocarcinoma, which is already manifested by the extreme heterogeneity of the disease, represents a difficult yet highly motivating challenge to address.

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Conflicts of interest

The authors declare no conflicts of interest.

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- of special interest
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VI.2 Annex II

RESEARCH LETTER

Peroxiredoxin-I
Sustains Inflammation
During Pancreatitis

Acute pancreatitis is a transient and local inflammation of the pancreas characterized by immune cell infiltration, fibrosis, and edema.¹ It mainly affects acinar cells, causing acinar metaplasia, and thereby constitutes a favorable environment for the development of pancreatic cancer in human beings and mouse models.² Despite the significant involvement of redox-dependent mechanisms in pancreatitis (eg, mitogen-activated protein kinase signaling, autophagy, disulfide stress, calcium signaling), supplementation with generic antioxidants is therapeutically unsuccessful,³ highlighting the need to identify specific targets amenable to pharmacologic therapy.

To identify redox targets relevant to pancreatitis, we first compared the transcriptional landscape of Fluorescence-activated cell sorting (FACS)-sorted acinar cells from control and cerulein-treated mice (cerulein is a pancreatitis-inducing compound). We identified an increased expression of activators of the peroxiredoxin pathway such as peroxiredoxin-1 (*Prdx1*), sulfiredoxin (*Srxn1*), and thioredoxin (*Txn1*) (Supplementary Figure 1A). Among the typical 2-cystein family members, mouse and human peroxiredoxin-1 protein (PRX-I), -II, -III, and -IV, only the expression of PRX-I was selectively induced in metaplastic acinar cells, at advanced stages of acute pancreatitis (Figure 1A and Supplementary Figure 1B-G). Accordingly, in primary human acinar cells cultured under conditions that mimic pancreatitis-induced metaplasia, we found substantially higher levels of PRX-I in metaplastic cells (days 3–4) compared with normal acini (day 0) (Figure 1B and Supplementary

Figure 1E and F). PRX-I has been shown to interact with inflammatory factors, such as nuclear factor κ B (NF- κ B) and macrophage migration inhibitory factor, suggesting its involvement in the pathophysiology of pancreatitis.⁴ To investigate the role of PRX-I in pancreatitis, we genetically ablated its expression using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) (Supplementary Figure 2A–D). This constitutive inactivation recapitulates the clinical context in which a drug administered to patients would inhibit its target in all cell types. Although a previous report showed that long-term constitutive PRX-I deletion causes anemia and a shortened lifespan,⁵ we did not observe any pancreas-specific anomaly in *Prdx1*^{−/−} mice (age, 3 mo). *Prdx1*^{−/−} mice were born at the expected Mendelian frequency, showed normal postnatal development, and were fertile.

Next, we analyzed the histology of pancreata from *Prdx1*^{+/+}, *Prdx1*^{+/-}, and *Prdx1*^{−/−} mice treated with cerulein in early and late acute settings (Supplementary Figure 2E and F). At early acute pancreatitis time points, *Prdx1*^{+/+} and *Prdx1*^{+/-} pancreata (considered together as controls [Ctrl]) showed a slight increase in PRX-I expression (Supplementary Figure 3A and B). The extent of edema and immune infiltration observed in Ctrl pancreata was not affected in *Prdx1*^{−/−} mice; the low PRX-I expression, at early pancreatitis, probably explains the minimal effects observed after its genetic ablation (Supplementary Figure 3C and D). Interestingly, at late acute pancreatitis, PRX-I expression was strongly increased in metaplastic acini (Supplementary Figure 3E and F). At this time point, pancreata from *Prdx1*^{−/−} mice showed a well-preserved architecture with a significantly 2-fold higher number of normal acini and a

3-fold reduction in metaplastic area compared with Ctrl (Figure 1C and D and Supplementary Figure 4A and B). CD45-positive immune cell infiltration and collagen deposit both were decreased significantly by 2-fold in *Prdx1*^{−/−} compared with Ctrl mice (Figure 1C and D).

PRX-I usually is described as an antioxidant enzyme with high catalytic efficiency.⁶ Interestingly, the content of protein carbonyls and 4-hydroxynonenal (4-HNE)-protein adducts was comparable in pancreata from Ctrl and *Prdx1*^{−/−} mice (Figure 1E). This suggested that the antioxidant function of PRX-I is not playing a predominant role in pancreatitis, which prompted us to search for additional roles of PRX-I. Previous reports have shown that PRX-I can be secreted from cultured cells in response to inflammatory stimuli and can bind to Toll-like receptor 4 to activate NF- κ B-mediated production of proinflammatory cytokines.^{7–9} Accordingly, we detected PRX-I in the culture medium of primary mouse acinar cells undergoing metaplasia, highlighting their ability to release PRX-I (Figure 2A). Strikingly, primary mouse acinar cells treated with recombinant PRX-I protein released significantly more proinflammatory cytokines interleukin 6 and tumor necrosis factor- α compared with untreated cells (Figure 2B). In line with this result, *Prdx1*^{−/−} pancreata showed a reduced expression of interleukin 6 and tumor necrosis factor- α , in the interstitial space between acinar cells, compared with their Ctrl counterparts (Figure 2C and D). Similarly, the expression and nuclear translocation of signal transducer and activator of transcription 3 and NF- κ B (subunit p65), 2 transcription factors controlling the expression of proinflammatory cytokines, were decreased by 2- to 3-fold in *Prdx1*^{−/−} pancreata (Figure 2C and D and Supplementary Figure 4A). Thus, our findings show

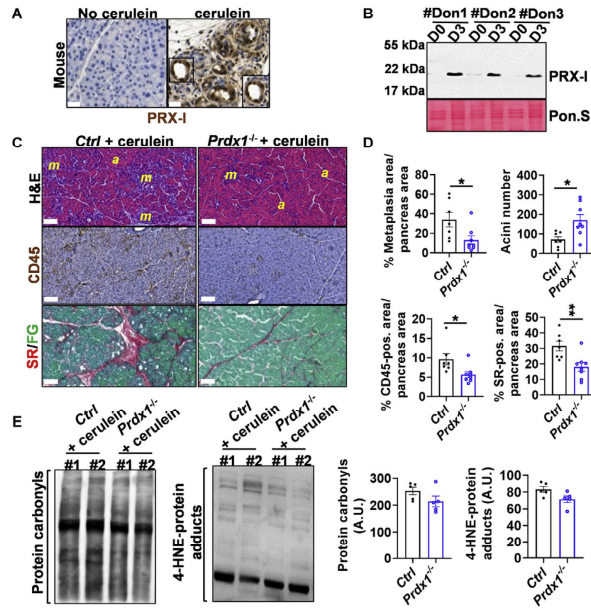
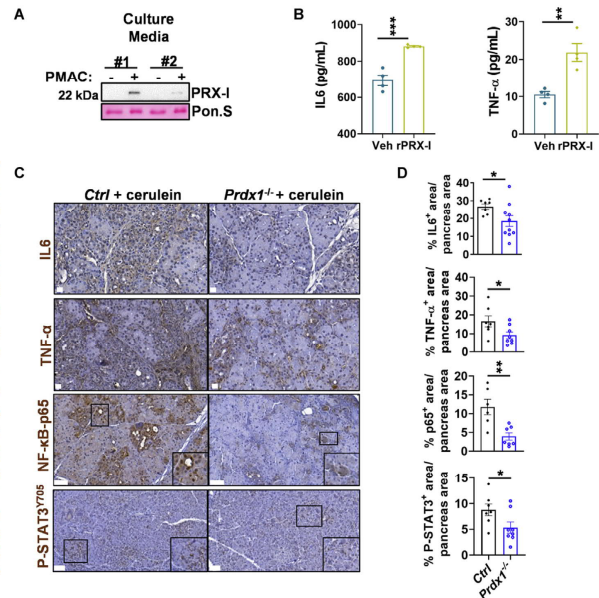


Figure 1. Ablation of PRX-I reduces the severity of late acute pancreatitis. (A) PRX-I staining on pancreas sections from mice treated or not with cerulein ($n = 3$). (B) Western blot for PRX-I performed on human pancreas culture lysates ($n = 4$). Day 0 (D0), normal acini; D3, metaplastic acini. Ponceau (Pon). S was used as loading control. (C) Histologic analysis on pancreas sections from cerulein-treated Ctrl ($n = 7$) and *Prdx1*^{-/-} ($n = 8$) mice. Scale bars: 50 μ m. (D) Whole-tissue quantification for data shown in panel C. (E) Similar levels of oxidative damages, protein carbonyls, and 4-hydroxynonenal (4-HNE)-protein adducts detected by Western blot on pancreatic lysates from cerulein-treated Ctrl and *Prdx1*^{-/-} mice ($n = 5$). The corresponding densitometry quantifications are also available. Data are means $\pm$ SEM. Statistical significance was tested by the Student *t* test: * $P < .05$; ** $P < .01$. Ctrl: *Prdx1*^{+/+} and/or *Prdx1*^{+/+}; a, normal acinar area; Don, donor; FG, Fast Green; m, metaplasia area; Pos, Positive; SR, Sirius Red.

Figure 2. PRX-I promotes the production of proinflammatory cytokines. (A) Western blot analysis for released PRX-I in culture medium in the presence or absence of primary mouse acinar cells (PMACs) undergoing metaplasia. PMACs were cultured for 2 days ($n = 3$). Ponceau (Pon). S was used as loading control. (B) Enzyme-linked immunosorbent assay for interleukin (IL)6 and tumor necrosis factor- α (TNF- α) on the culture medium of metaplastic acinar cells treated with vehicle (Veh) (0.02 mol/L HEPES, pH 7) or recombinant PRX-I (rPRX-I) (100 nmol/L) for 1 day ($n = 4$). (C) IL6 and TNF- α (scale bars: 20 μ m), as well as Phospho-signal transducer and activator of transcription 3 (P-STAT3^{Y705}) and NF- κ B (p65) (scale bars: 50 μ m) immunostaining on pancreata from cerulein-treated Ctrl ($n = 6-7$) and *Prdx1*^{-/-} ($n = 7-10$) mice. (D) Whole-tissue quantification for data shown in panel C (+, positive). Data are means $\pm$ SEM. Statistical significance was tested by the Student *t* test: * $P < .05$, ** $P < .01$, and *** $P < .001$. Ctrl: *Prdx1*^{+/+} and/or *Prdx1*^{+/+}.



that a mechanism linking the secretion of PRX-I to the production of proinflammatory cytokines may operate in vivo.

In summary, we discovered that the ablation of PRX-I reduces the severity of inflammation and related acinar-to-ductal metaplasia (Supplementary Figure 4C). Our results support PRX-I as a potential therapeutic target to reduce pancreatic inflammation and related damage.

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Abbreviations used in this letter: Ctrl, control; NF- κ B, nuclear factor κ B; PRX-I, mouse and human peroxiredoxin-1 protein

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Conflicts of interest

The authors disclose no conflicts.

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