Biliary tract cancer: molecular biology of precursor lesions

(Seminars in Liver Disease 43, 472-484, 2023)

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Abstract

Biliary tract cancer is a devastating malignancy of the bile ducts and gallbladder with a dismal prognosis. The study of the precancerous lesions has received considerable attention and led to a histopathological classification which, in some respects, remains an evolving field. Consequently, increasing efforts have been devoted to characterize the molecular pathogenesis of the precursor lesions, with the aim of better understanding the mechanisms of tumor progression, and with the ultimate goal of meeting the challenges of early diagnosis and treatment. This review delves into the molecular mechanisms that initiate and promote the development of precursor lesions of intra- and extrahepatic cholangiocarcinoma and of gallbladder carcinoma. It addresses the genomic, epigenomic and transcriptomic landscape of these precursors, and provides an overview of animal and organoid models used to study them. In conclusion, this review summarizes the known molecular features of precancerous lesions in biliary tract cancer and highlights our fragmentary knowledge of the molecular pathogenesis of tumor initiation.

Keywords: Cholangiocarcinoma; Gallbladder cancer; Tumor progression; Animal model; Organoid.

Lay summary. Cancers affecting the bile ducts and gallbladder still have a grim prognosis. While much effort has been devoted to characterize the molecular biology of the invasive lesions at a late stage of the disease, less is known about the precancerous lesions and about the molecular anomalies that promote progression toward cancer. Here we review the different kinds of precancerous lesions and describe the most frequent genetic alterations and

their evolution during tumor progression. We also describe how the use of animal models and patient-derived organoids can contribute to a better understanding of tumor initiation and progression in biliary tract cancers.

Introduction

Biliary tract cancers are rare tumors which are receiving much attention in recent years as a result of their increased incidence and high mortality rate[1,2]. Gallbladder cancer (GBC) is the most frequent biliary tract cancer, with adenocarcinoma being the most common histological subtype, representing more than 90% of cases [3,4]. There is a female predominance in GBC incidence rates. The major risk factor of GBC is chronic inflammation, predominantly caused by the presence of gallstones. Other diseases provoking chronic inflammation and potentially leading to GBC include primary sclerosing cholangitis, chronic carriage of *Salmonella* or *Helicobacter*, and exposure to toxins [5]. Pancreaticobiliary maljunction also increases the risk of GBC as it promotes inflammation by favoring a reflux of pancreatic secretions toward the biliary tract [6]. Bile duct cancers are usually referred to as cholangiocarcinomas. They are classified according to their anatomical location into intrahepatic cholangiocarcinoma (iCCA) at the periphery of the second-order bile ducts, perihilar cholangiocarcinoma arising in the right and/or left hepatic duct and/or at their junction, and distal cholangiocarcinoma. The latter two are often grouped together as extrahepatic cholangiocarcinoma (eCCA). Like GBC, iCCA develops as adenocarcinomas, yet with specific histological features depending on their location in small (non-mucin-secreting iCCA) or large ducts (mucin-secreting iCCA). eCCA and large duct iCCA share most histological characteristics [7]. Small duct iCCA, large duct iCCA and eCCA have different etiologies. For instance, viral infection (hepatitis B and C), non-alcoholic fatty liver disease and cirrhosis promote small duct iCCA, whereas liver fluke and primary sclerosing cholangitis are associated with large duct iCCA and eCCA. Although a significant number of patients do not present with known risk factors, chronic inflammation caused by infections, immune reaction, cholelithiasis, environmental toxins (asbestos), or malformations (*e.g.* Caroli disease, choledochal cysts) is a hallmark of cholangiocarcinoma [8].

The histology and etiology of biliary tract cancers suggest that common and region-specific pathogenic mechanisms may be at play to induce the various types of biliary tract cancers. Along the same lines, the transcriptome of the cholangiocytes, which form the biliary epithelium from which most tumors emerge, is characterized by a common core signature associated with regional-specific expression of a subset of genes. Cholangiocytes display significant functional, transcriptional and morphological heterogeneity along the biliary tract, and originate from distinct regions of the embryonic endoderm [9-13]. Recent single-cell analyses compared the cholangiocytes lining the intrahepatic ducts, the common bile duct and the gallbladder, and demonstrated that their transcriptional profile largely depends on microenvironmental cues and exposure to bile while maintaining a core transcriptional network [14]. Here we will discuss the molecular mechanisms initiating biliary tract cancer and progression toward malignancy, while focusing on GBC and cholangiocarcinoma precursor lesions, and underlining common and regional characteristics. We refer to excellent recent reviews for a full account on the molecular genetics of full-blown malignant tumors, and for an in-depth description of the histopathology of the precursor lesions[4,8,15-19].

Histogenic sequence of gallbladder cancer and cholangiocarcinoma

Gallbladder cancer. Two pathways are considered to lead to GBC. The first is characterized by the sequential development of metaplasia, dysplasia and carcinoma (Fig. 1A). This sequence was already suggested 30 to 40 years ago and represents the majority of diagnosis, being typically associated with gallstone disease [20-22]. In this context, metaplasia and dysplastic lesions are considered precursor lesions of GBC. Metaplasia is histologically classified as intestinal, foveolar, or pseudopyloric (antral), the latter being less susceptible to evolve to cancer (Fig. 1A) [23]. Intestinal metaplasia appears as patches of intestinal-like

epithelium (enterocyte-like) interspersed with goblet cells (Fig. 1B) and sometimes with Paneth cells. The morphologic appearance of pseudopyloric metaplasia is reminiscent of epithelial cells of the gastric pyloric antrum, and foveolar metaplasia displays histological features of gastric foveolar type surface epithelium. [23,24]. Dysplasia consist of biliary intraepithelial neoplasia (BilIN) and appear as microscopic, flat or micropapillary lesions, which are ranked as low-grade or high-grade [24-26]. Four histological subtypes of BilIN are identified, namely pancreatobiliary, oncocytic, gastric and intestinal (Fig. 1A). Low-grade BilINs have preserved nuclear polarity, hyperchromatic and pseudostratified nuclei, moderate cytoarchitectural atypia and increased nucleo-cytoplasmic ratio, whereas high-grade BilINs, also known as carcinoma *in situ*, display loss of nuclear polarity, pleomorphic nuclei with complex stratification and micropapillae [17,27,28]. High-grade BilINs are often detected in the neighborhood of biliary tract cancers, and are therefore considered an immediate precursor of the malignant lesions. However, how GBC develops from metaplasia and dysplasia is currently questioned, as discussed below.

The second histogenic pathway in GBC pathogenesis follows an adenoma → carcinoma sequence and concerns 5-10% of GBC [29] (Fig. 1A). There is some debate about the classification of gallbladder adenoma. In the most recent classification of digestive system tumors by the World Health Organization, pyloric gland adenoma (PGA) is separated from the other adenomas which are grouped together as intracholecystic papillary neoplasms of the gallbladder (ICPN) [19,23,30] (Fig. 1A). The histopathology of ICPN is characterized by a macroscopically visible mass-forming tumor which displays distinct histological patterns defined as biliary, gastric, intestinal or oncocytic [31]. Like for BilINs, a two-tiered grading system distinguishes low-grade from high-grade ICPNs, based on their degree of cytoarchitectural atypia. Approximately 50% of ICPNs are associated with GBC, strongly

suggesting that ICPNs are premalignant. This suggestion was recently supported by an ultrasound follow-up of a patient who had a papillary lesion of the gallbladder that evolved from benign to malignant within a 2-year period and which displayed histological criteria of ICPN upon resection [32]. The occurrence of ICPN is less clearly correlated with the presence of cholelithiasis, but ICPNs are often detected in combination with pancreaticobiliary maljunction [33].

PGA is another precursor of GBC. It presents as a grossly visible noninvasive neoplasm composed of mucinous glands arranged in a tubular configuration, and is also graded as low- or high-grade. Finally, mucinous cystic neoplasm is also considered a precursor lesion of GBC but is of minor importance as compared to BilIN and ICPN, and will not be discussed further.

Cholangiocarcinoma. BilINs are precursors common to GBC and cholangiocarcinoma (Fig. 2). In the latter they are detected in the extrahepatic and large intrahepatic ducts, and originate from the mucosa, possibly also from peribiliary glands. Low-grade BilINs are at risk for transition to high-grade lesions, and the frequent location of high-grade BilINs near carcinoma lesions suggests that high-grade BilINs are direct precursors of cancers.

Intraductal papillary neoplasms of the bile duct (IPNB) affect the extrahepatic and large intrahepatic bile ducts and, in contrast to BilINs, are macroscopically detectable as a mass-forming or polypoid papillary lesion [34] (Fig. 2A-B). They originate from the mucosa and possibly from peribiliary glands in a context of chronic inflammation caused by hepatolithiasis, parasite infections, congenital biliary tract malformations or exposure to environmental toxins. Like for gallbladder ICPN, IPNBs present as 4 differentiation subtypes

(intestinal, gastric, pancreatobiliary and oncocytic) and are categorized as low-grade and high-grade based on the level of nuclear atypia, cellular pleomorphisms and polarity (Fig. 2A) [18]. Considering that the criteria to define low-grade or high-grade IPNB were not well adopted by all pathologists, an alternative classification into types 1 and 2 IPNB was proposed. This alternative approach considers structural aspects of IPNB (location, stromal invasion, heterogeneity, regularity, fibrovascular stalks) in addition to cytological criteria, and defines type 1 lesions as composed of regularly-structured low-grade and high-grade IPNBs, and type 2 lesions as irregular and heterogeneous high-grade IPNBs. This classification was evaluated on a large number of pathological samples and it was concluded that type 1 lesions were likely precursors of type 2 and of unclassifiable IPNBs [35]. In parallel, another classification distinguishes 5 subtypes of IPNB, namely intestinal, gastric, and pancreatobiliary subtypes, as well as intraductal oncocytic papillary neoplasm (IOPN) and intraductal tubulopapillary neoplasm (ITPN) [36]. ITPNs are increasingly recognized as a separate entity, lacking intestinal differentiation, expressing MUC6, and forming tubular configuration [37]. Finally, mucinous cystic neoplasm is also considered a precursor to iCCA and eCCA, yet it is of minor importance and will not be analyzed in this review.

Molecular biology of precursor lesions

Gallbladder cancer. The metaplasia → low-grade BilIN → high-grade BilIN → carcinoma histogenic sequence has been investigated at the genetic level. Many studies report cases in which precursor lesions and GBC were sampled in distinct patients. Therefore, the molecular cascade that was deduced from these analyses provides a global view of the pathogenic cascade, but does not necessarily reflect the tumorigenic process taking place in individual patients. Also, the establishment of a clear relationship between specific gene alterations and specific stages of tumor progression did not emerge easily because the analysis of distinct

patient cohorts revealed distinct mutational profiles [38]. Still, *TP53* loss-of-functions are frequent early events in GBC pathogenesis, consistent with the fact that *TP53* alterations are often associated with genomic instability and DNA damage caused by chronic inflammation, which is a major risk factor for GBC [39]. A progressive increase in TP53 protein overexpression was observed when comparing low-grade BilIN, high-grade BilIN and GBC, while no difference was found between normal epithelium and metaplasia [40]. In this study, the mutational status of *TP53* was not determined. Other genetic changes detected in gallbladder BilIN lesions affect *CTNNB1*, *ARID2*, *ERBB3*, *PI3KCA*, *APC* and *SMAD4* (Fig. 3A) [41]. *CTNNB1* mutations were predicted to be damaging, but the activity of β-catenin protein was not characterized. Beyond the precursor stage, additional mutations accumulate, and in adenocarcinoma, the most frequent mutations affect the *KRAS, CTNNB1, TP53*, *PI3KCA*, *ERBB2*, *CDKN2A* and *CDKN2B* genes[16,42-49]. In adenocarcinoma *TP53* mutations are distributed across the gene and the tumors express activating *CTNNB1* mutants, indicating that the β-catenin pathway contribute to cancerogenesis.

The metaplasia → dysplasia → carcinoma tumorigenic sequence was recently challenged by Lin and coworkers who microdissected and exome sequenced patient samples which concomitantly displayed GBC, low-grade and high-grade BilIN lesions, as well as normal gallbladder tissue [41]. The concomitant presence of precursor and malignant lesions within a same patient significantly increases the possibility that the former has given rise to the latter, in particular when both lesional types share molecular characteristics. Based on single nucleotide variant analyses, evolutionary trees of tumor progression were generated, leading to the conclusion that two pathogenic groups of GBC could be distinguished. In the first group, GBC split before the common ancestor of low-grade and high-grade BilINs, whereas in the second group, GBC split after the common ancestor of BilINs. Consequently, the

evolution of the two groups is regarded as BilIN-independent or BilIN-dependent, respectively. Loss-of-heterozygosity is known to accumulate in GBC precursor lesions [50], and the two groups also differed by the number of loss-of-heterozygosity and mutational events, which were more numerous and occurred earlier during development of BilIN-independent GBC. Out of these two tumor progression scenarios, only the BilIN-dependent group fits well with the notion of a metaplasia → dysplasia → carcinoma histogenic sequence. Our own results support and extend these conclusions at the transcriptome level: spatial transcriptomic analyses of samples which simultaneously displayed multiple areas of normal epithelium, BilINs and adenocarcinoma suggest that adenocarcinoma can derive from high-grade or low-grade BilIN (unpublished) (Fig. 3A).

Further, Lin and coworkers showed that tumor mutation burden was similar between lesions within a same patient, and that the number of mutations increases with age [41]. Importantly, the mutations did not differ much between precursor and malignant lesions of a same patient, supporting that the precursors have evolved toward GBC. Instead, the mutational profile varied between patients, supporting the existence of patient-specific mechanisms of tumor formation. Such patient-specificity was also found in our spatial transcriptomics analysis which revealed that the sequence of signaling pathway activation during tumor progression differs significantly between patients (unpublished). Interestingly, 50% of the patients studied by Lin and coworkers bore missense *CTNNB1* mutations. These mutations were found in all lesional types, both in BilIN-dependent and BilIN-independent processes [41], indicating that *CTNNB1* can be mutated early in the tumorigenic process while not imposing a BilIN-dependent or -independent pathway.

The accumulation of mutations during cancerogenesis causes the development of subclonal cell populations that have distinct properties, ultimately leading to the selection of subclones with proliferative and invasive advantage. Such subclonal selection has now been detected at the BilIN stage, with some subclones shrinking during tumor progression while others expand and branch during BilIN evolution and their transition to cancer [51].

In part due to its relative rarity, the adenoma → carcinoma sequence is less studied at the genetic level. Targeted sequencing identified *CTNNB1* mutations in more than half of PGAs suggesting that such mutations are a hallmark of PGA [52-54]. β-catenin protein accumulated in the nucleus of the cells. The analysis of 7 ICPNs revealed frequent mutations in *STK11*, *CTNNB1*, and *APC* [55] (Fig. 3A). The latter two, combined with frequent detection of β-catenin in the cytoplasm, suggest an early activation of the β-catenin pathway, but this was not confirmed in a distinct cohort [17]. No *TP53* mutation was found in another patient, but a high proportion of studied cases (4 out of 10) showed activating *KRAS* mutations [56,57]. The low number of *TP53* mutations and the higher number of *KRAS* mutations in ICPNs contrast with the findings in BilINs and suggest that the mutational sequence in the adenoma → carcinoma pathogenic path differs from that in the metaplasia → dysplasia → carcinoma histogenic sequence. However, the limited number of studied ICPNs imposes some care. Genomic investigations of GBC associated with pancreatobiliary maljunctions revealed the frequent occurrence of mutations in *TP53*, *EGFR*, *RB1*, *ERBB2*, *MET*, *PTPN1* and *KDR* at the GBC stage [58]. When these mutations arose during tumor progression and whether part of the GBC evolved from ICPNs is unknown.

GBC tumorigenesis was also investigated at the epigenomic level. Cumulative changes in promoter methylation were detected during progression from cholecystitis to cancer,

revealing that aberrant methylation is an early event in GBC pathogenesis [49,59-63]. Digging deeper into the epigenomics of gallbladder BilINs and carcinoma, Brägelmann and coworkers found a progressive hypermethylation of CpG islands and gene promoters, mainly in high-grade dysplasia and GBC [64]. This affected negative regulators of Hedgehog signaling (*ZIC1*, *ZIC4*, *HHIP*, *PTCH1*), Wnt signaling (*APC*), and tumor suppressors (*WIF1*, *RUNX3* and *TP73*) (Fig. 3A). Copy number analysis revealed that the frequency of copy number alterations increases along the metaplasia → dysplasia → GBC sequence, and often affects the tumor suppressors *CDKN2A* and *TP53*, as well as *MDM2*, a negative regulator of TP53 [64]. This provides additional support to the critical role of TP53 pathway alterations in the pathogenesis of GBC. Other genes affected include those that code for signaling ligands and effectors of the EGF-ERBB and FGF-FGFR families, an interesting observation which opens perspectives for the design of pathway-targeting therapy [64].

The genomic and epigenomic anomalies occurring during GBC tumor progression have their transcriptomic counterpart. Hypermethylated genes are repressed and hypomethylated genes are overexpressed, as illustrated by the progressively decreased expression of tumor suppressors (*CDKN2A, TP73*) and of negative regulators of Hedgehog (*HHIP*) and Wnt signaling (*APC*) (Fig. 3A) [64,65].

Cholangiocarcinoma. Genomic alterations were found in BilINs associated with cholangiocarcinoma. *KRAS* mutations likely represent early events, while *TP53* alterations are more often found in higher-grade lesions, like in gallbladder BilIN [66,67]. The study of protein expression revealed several features typical for perturbed control of cell proliferation, deregulated autophagy, and abnormal chromatin modification, as illustrated by the upregulation of CDKN1A, CyclinD1, LC3 and the Polycomb group protein EZH2, in parallel

with downregulation of SMAD4 [68]. At the transcript level, high-throughput miRNA profiling of BilINs in distal cholangiocarcinoma identified several dysregulated miRNAs, in particular miR-451a and miR-144-3p, which behave as tumor suppressors that inhibit cell migration [69]. The latter observation fits with the reduced expression of E-cadherin, another modification supporting enhanced migration [70].

Genetic analyses of IPNBs uncovered mutations in a large set of genes, with *KRAS*, *GNAS*, *TP53*, *SMAD4*, and *CDKN2A* being among the most frequently affected [36,71-73] (Fig. 3B). Since mutations may be preferentially associated with specific etiologies, several groups attempted to correlate the mutational profile with the histological subtypes. For instance, chronic liver fluke infections induce mainly pancreatobiliary IPNBs, and fluke-associated eCCAs display frequent inactivating mutations in *MLL3*, *ROBO2*, *RNF43* and PEG3, as well as activating mutations in *GNAS*, thereby affecting histone modification, genome stability and G protein signaling [18,74]. Although there is a lack of consensus on the classification of IPNBs, there is a significant overlap between the classifications, enabling us to draw fairly consistent conclusions about the most frequent mutations in each IPNB subtype (Table 1). Still, these conclusions need to be considered with care, in particular while taking into account that the mutational profile is influenced by the ethnical or geographical origin of the tumors, as exemplified by the low incidence of *GNAS* mutations in a European cohort as compared to a Japanese cohort [75,76]. Interestingly, in the latter cohort, activating *GNAS* mutations were more frequent in IPNBs than in carcinoma, which is reminiscent of pancreatic cancer tumorigenesis in which *GNAS* mutations are more frequent in precursor than in malignant lesions [75,76].

The evolution of the mutational profile during IPNB development was initially investigated by comparing low-grade IPNB with high-grade IPNB, and type 1 with type 2 IPNBs. This revealed a higher occurrence of *TP53* mutations and TP53 nuclear staining in high-grade and

type 2 lesions than in low-grade and type 1 lesions, whereas *KRAS* mutations were more frequent in low-grade and type 1 IPNBs [71,72,82]. Along the same lines, anomalies of protein expression found in high-grade IPNBs (overexpression of HER2, EGFR, cyclinD1, EZH2 and p53; loss of SMAD4 detection) but not -or less frequently- in low-grade lesions were interpreted as evidence for a tumor progression mechanism [70,82,83]. However, most of these initial data compared precursor lesions of various grades that were not concomitantly found in the same patient, which prevents us to characterize the pathogenic path in individual patients. Recent work by Goeppert and coworkers addressed this issue in detail in a large set of patient samples harboring simultaneously cholangiocarcinoma and IPNB or ITPN precursor lesions [73]. Their results nicely illustrate that a large set of mutations are detected both in the precursor and invasive lesion, supporting that the invasive cholangiocarcinoma developed from the precursor IPNB or ITPN. IPNB and ITPN displayed a different mutational landscape. Key oncogenic pathways in ITPN related to chromatin remodeling, cell cycle regulation and DNA damage-repair, as evidenced by mutations affecting these pathways. Whole exome sequencing of ITPNs detected frequent alterations in *TG*, *FGFR2*, *AMER2*, *SLIT2*, *HMCN1* and *CEP17*, in addition to the *HIST2H3C/D* and *ATM* genes pointed out by Wang and coworkers [36,73,84] . More than half of the studied ICPN bore mutations in ligands or effectors of Wnt signaling. The nuclear accumulation of β-catenin further supported that canonical Wnt signaling was activated.

Interestingly, while the incidence of *CTNNB1*, *CDKN2A* and *SMAD4* mutations decreased during tumor progression from IPNB, that of *ROBO1*, *ROBO2* and *FBXW7* increased (Fig. 3B), suggesting that the latter contribute to trigger transition toward malignancy and that the former do no longer confer a proliferative advantage once the tumor has reached a malignant stage [73]. The lack of nuclear accumulation of β-catenin in cancer lesions extended the conclusion that Wnt signaling was no longer essential in carcinoma [80,82,85].

These findings are in line with the subclonal evolution of gallbladder BilINs mentioned above [51]. In that context, we speculate that those mutations which drive IPNB development but are no longer present in cholangiocarcinoma reflect that the cell subclone bearing the mutations disappears as a result of competition with subclones that have a proliferative advantage. Alternatively, the pathways triggered by these mutations may undergo an epigenetic switch characterized by irreversible bistability. Once the pathway activity has been triggered by the mutation, the disappearance of the trigger is not sufficient to allow the pathway to restore its initial normal activity. The evolutionary pressure to maintain the mutation would then disappear, allowing the development of cell clones that are devoid of the mutation but still display high pathway activity [86].

At the epigenomic level, Schlitter and coworkers noticed a significant proportion of patients with hypermethylation of the tumor suppressor gene *CDKN2A* in low- and high-grade IPNB, which parallels the higher expression of *DNMT1* in type 2 IPNB as compared to type 1 IPNB [82,87]. Genome-wide DNA methylation profiling uncovered that paired precursor lesions and corresponding invasive cholangiocarcinoma coexisting in patients exhibit similar DNA methylation profiles [73]. This profiling further revealed three distinct clusters, comprising (i) IPNB and distal cholangiocarcinoma, (ii) ITPN, perihilar and intrahepatic cholangiocarcinoma, and (iii) normal biliary epithelium, further supporting that ITPN and IPNB represent distinct pathogenic entities. Notably, a detailed analysis of the methylation profile led to the proposal that ITPNs share a common cell-of-origin. In contrast, IPNB may derive from diverse cell-of-origin types, suggesting that ITPNs and IPNBs originate from different cellular origins [73].

Animal models for studying precursor lesions of biliary tract cancer

To unravel the molecular mechanisms driving the formation of cholangiocarcinoma, animal models have been instrumental. They were designed based on administration of carcinogens, induction of chronic cholestasis, grafting of tumor cells and induction of genetic alterations mimicking those occurring in patients [88-91]. Genetically engineered mouse models of cholangiocarcinoma typically induce oncogenic mutations or repress the function of tumor suppressors in cholangiocytes, hepatocytes or embryonic hepatoblasts using a Cre-loxP strategy. Cre-mediated induction of gene alterations in cholangiocytes meets with variable efficiency of the Cre driver in intrahepatic, extrahepatic or gallbladder cholangiocytes. Tamoxifen-inducible *CK19-Cre^{ERT}* and *Hnf1β-Cre^{ERT2}* can successfully induce recombination of loxP-flanked gene regions in intrahepatic, extrahepatic or gallbladder cholangiocytes [92-94]. *OPN-Cre^{ERT2}* is more efficient than *CK19-Cre^{ERT}* in intrahepatic cholangiocytes [93], but has nearly no detectable activity in cholangiocytes of the gallbladder epithelium (unpublished). So far, the most widely used and best characterized Cre lines allowing gene recombination in cholangiocytes are *CK19-Cre^{ERT}* and *Hnf1β-Cre^{ERT2}* for gallbladder, intra- and extrahepatic cholangiocytes, and *OPN-Cre^{ERT2}* for intrahepatic cholangiocytes. The initial goal of mouse models was to induce malignant tumors, and overall less attention has been paid to the precursor lesions. Table 2 lists those genetically-engineered mouse models of cholangiocarcinoma and GBC in which the appearance of precursor lesions has been documented.

The tumorigenic pathway of IPNB was investigated in more detail in three sets of mouse models. First, FGF10 signaling is activated in approximately half of IPNBs and using an impressive list of mouse models, Tomita and coworkers elegantly demonstrated that autocrine or paracrine overexpression of FGF10 induces precancerous intrahepatic and extrahepatic IPNB and is required to maintain the lesions [102]. This was associated with the activation of the

KRAS-MEK and SRC pathways. Second, another pathway involving a *Kras*^{G12D} → *Sox17* → *Tns4* cascade in an inflammatory context was shown to drive development of intrahepatic IPNB and their evolution to iCCA [100]. Third, constitutive activation of KRAS signaling and canonical Wnt signaling in cholangiocytes was sufficient to induce extrahepatic BilIN and gastric-type ICPN in gallbladder [94]. In this study, the premature death of the mutant mice prevented evolution until the cancer stage. However, spheroids derived from the mutant epithelia generated adenocarcinoma after subcutaneous injection into immunodeficient mice, demonstrating a malignant potential. The tumors were characterized by the accumulation of new mutations and their development required the maintenance of functional TGFβ signaling [94].

Development of BilINs in the extrahepatic bile ducts and gallbladder was noticed in mice with cholangiocyte-specific and constitutive activation of the Notch pathway when this was combined either with *Pten* loss-of-function or with induction of oncogenic *Kras*^{G12D} [101]. In both cases, this induced downstream stimulation of *Myc* expression, which resulted in derepression of the mTORC1 pathway, which was then found to correlate with NOTCH1 activity in eCCA and GBC, and to be required for growth of biliary cancer cells.

Nongenetic mouse models have the advantage of considerably facilitating the breeding of the animals. In that context, Rosa and coworkers fed wild-type C57BL/6 mice with a high-cholesterol lithogenic diet [106]. The mice developed gallstones, and gallbladder metaplasia were detected within a month. Within 9 months, 60% of the animals evolved toward dysplasia. No carcinoma was observed. This model was not only useful to demonstrate the efficacy of a cholesterol absorption inhibitor as a preventive drug, but it also suggested that combining genetic changes with a high-cholesterol lithogenic diet could constitute excellent

models to recapitulate the human metaplasia → dysplasia → GBC sequence. Along the same lines, wild-type rats have been used to investigate the metaplasia → dysplasia → cholangiocarcinoma sequence by exposing the animals to toxins. Chronic oral administration of thioacetamide elicited progressive evolution of the intrahepatic biliary epithelium toward intestinal-type metaplasia, followed by dysplasia and iCCA characterized by overexpression of *Erbb2* and *Met* starting at the dysplastic stage [107]. Finally, zebrafish is also used as model to investigate cholangiocarcinoma [90]. To the best of our knowledge, precursor lesions have not been studied in this model.

Biliary tract precursor-derived organoids

Organoids are in *vitro* three-dimensional models able to self-organize by forming cell-cell and cell-matrix interactions mimicking diverse tissue-specific functions and structures, making them useful for studying diseases [108]. Biliary tract precursor-derived organoid lines could provide valuable insights into the study of early tumorigenic states, the identification of biomarkers and potential treatments. However, few authors have been able to establish such precursor-derived organoids to date. Recently, organoids were derived from benign gallbladder adenoma [109]. The organoids exhibited irregularly shaped cystic cribriform structures, and displayed histology and gene expression similar to those of the parental tissue. Their use to study tumor progression awaits further investigation.

In contrast, several groups have successfully derived organoids from malignant GBC and cholangiocarcinoma [109-112]. Nevertheless, they reported the importance of adapting the established protocols, since non-malignant cholangiocytes are able to expand and take over the cultures. Therefore, confirming that the established organoids have originated from cancer

cells is of critical importance. Similar efforts have been made when developing organoids from other organs, highlighting the importance of adapting the isolation protocol to the desmoplasticity of the sample or to its mutational spectrum. The remarkable work of Fujii and coworkers illustrates how the growth factor requirement needs to be optimized to establish colorectal organoids derived from distinct histological subtypes and clinical stages [113]. They reported that some factors are critical to the growth of specific organoid types while exhibiting detrimental effects on others. For instance, the propagation of adenoma organoids was done in the absence of Wnt-promoting factors (Wnt3A/R-spondin1) due to the fact that all of them harbored mutations within the Wnt signaling pathway. Wnt-promoting factors are commonly used in the derivation of biliary tract organoids [114], and Yuan *et al.* reported the upregulation of Wnt signaling in gallbladder adenoma-derived organoids, as compared to those derived from cancer samples [109]. As mentioned above, biliary tract precancerous lesions often present with mutations in Wnt signaling-related genes, and mutations in *CTNNB1* have been reported in early stages of gallbladder metaplasia. Therefore, we hypothesize that the success in the derivation of biliary precursor-derived organoids will depend on the adaptation of the growth factor requirements to the mutational spectrum of the samples.

Concluding Remarks

Gallbladder cancer and cholangiocarcinoma exhibit a stealthy progression from precursor lesion toward malignancy, underlining the critical need for tools to facilitate early diagnosis and intervention. Exploring the molecular pathogenesis of these precursor lesions is of great importance, offering crucial insights into the mechanisms that drive progression toward malignancy. Although significant progress has been made in matching molecular alterations to specific histopathological types of precursor lesions, our knowledge of molecular pathogenesis at the genomic, epigenomic and transcriptomic levels remains fragmentary.

Characterizing the proteome of precancerous lesions and their interaction with the surrounding mesenchyme and stroma are priorities for future research. The molecular characterization of patient-specific mechanism and the development of tools to address tumor-promoting mechanisms are still in their infancy.

Acknowledgements

The authors thank Dr. Sophie Pirenne for providing images of gallbladder tumor development. The work of F.P.L. is supported by the Fonds de la Recherche Scientifique-FNRS (Belgium, grant Télévie 7.8505.21), the Belgian Foundation against Cancer (grant #2018-078), and the Fonds Joseph Maisin (grants #2020-2021 and #2022-2023). F.M.-N. is supported by a post-doctoral fellowship from the Fonds de la Recherche Scientifique-F.N.R.S. (Belgium, Télévie #7.8505.21). L.P.T.A. is Research Fellow of the Fonds de la Recherche-F.N.R.S. Scientifique (Belgium, grant #1.A.424.24F).

Conflict of interest statement

None declared

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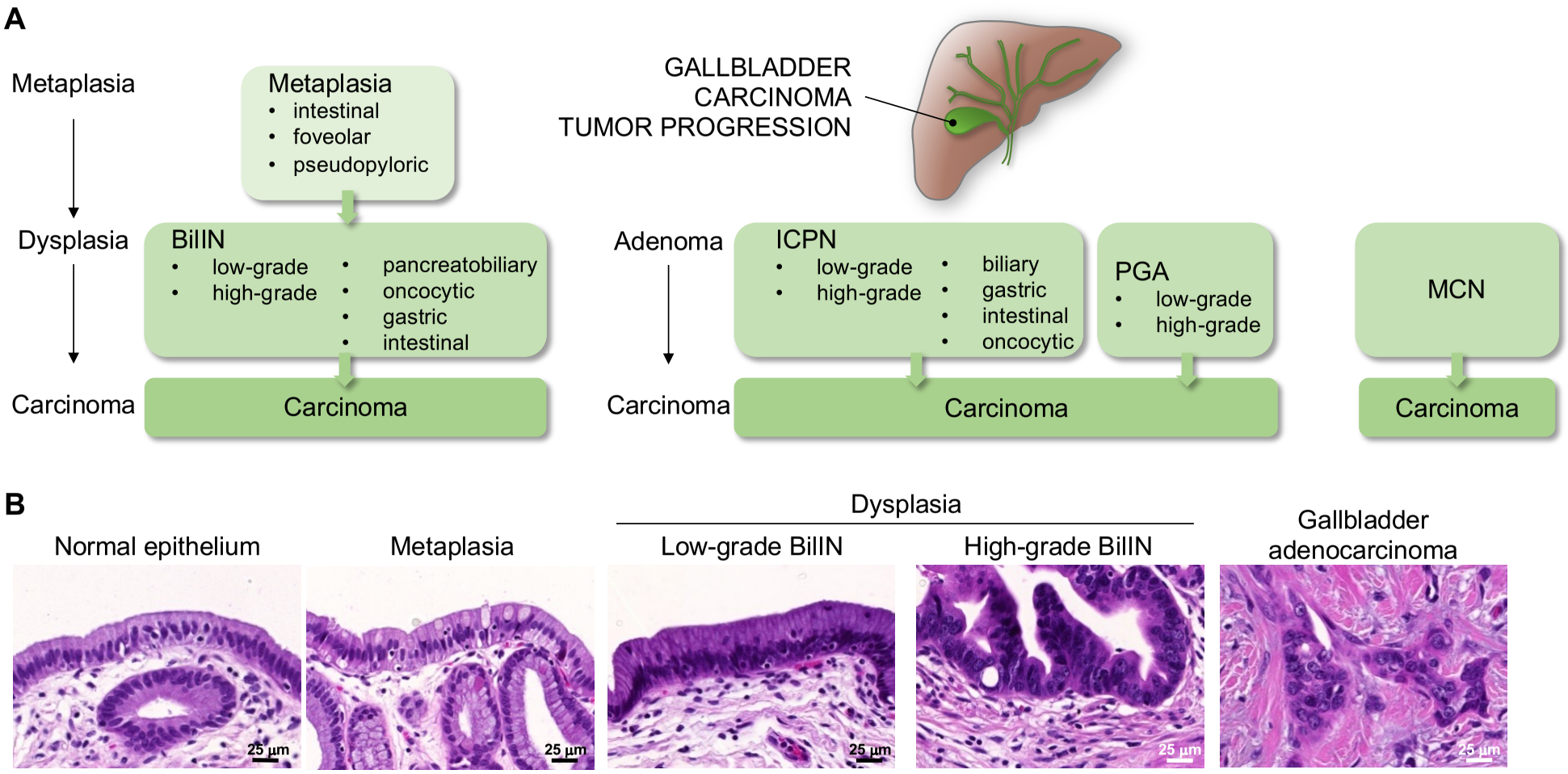
Figure captions

Figure 1. Histogenic sequences leading to gallbladder cancer. (**A**) Classification of precursor lesions of gallbladder cancer involved in metaplasia → dysplasia → carcinoma, and adenoma → carcinoma histogenic sequences. (**B**) Hematoxilin and eosin staining of section illustrating the metaplasia → dysplasia → carcinoma sequence in gallbladder tumor progression. Panels illustrating normal epithelium, dysplasia and carcinoma are from a same patient. The intestinal metaplasia characterized by the presence of goblet cells is from a distinct patient. BilIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasms of the gallbladder; IOPN, intraductal oncocytic papillary neoplasm; IPNB, intraductal papillary neoplasm of the bile duct; ITPN, intraductal tubulopapillary neoplasm; MCN, mucinous cystic neoplasm; PGA, pyloric gland adenoma.

Figure 2. Histogenic sequences leading to intrahepatic large duct type and extrahepatic cholangiocarcinoma. (**A**) Classification of precursor lesions of cholangiocarcinoma. (**B**) Hematoxilin and eosin staining of sections illustrating the normal → BilIN → carcinoma and normal → IPNB → carcinoma sequences in iCCA tumor progression. BilIN, biliary intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasms of the gallbladder; IOPN, intraductal oncocytic papillary neoplasm; IPNB, intraductal papillary neoplasm of the bile duct; ITPN, intraductal tubulopapillary neoplasm; MCN, mucinous cystic neoplasm; PGA, pyloric gland adenoma.

Figure 3. Most frequent molecular alterations occurring during tumor progression from precursor lesion to carcinoma. (**A**) Tumor progression in gallbladder cancer. (**B**) Tumor progression in cholangiocarcinoma. BilIN, biliary intraepithelial neoplasia; GBC, gallbladder cancer; eCCA, extrahepatic cholangiocarcinoma; iCCA, intrahepatic cholangiocarcinoma;

ICPN, intracholecystic papillary neoplasms of the gallbladder; IPNB, intraductal papillary neoplasm of the bile duct; ITPN, intraductal tubulopapillary neoplasm; PGA, pyloric gland adenoma.


A
Metaplasia
Dysplasia
Carcinoma
Metaplasia
• intestinal
• foveolar
• pseudopyloric
BilIN
• low-grade
• high-grade
• pancreatobiliary
• oncocytic
• gastric
• intestinal
Carcinoma
GALLBLADDER
CARCINOMA
TUMOR PROGRESSION
Adenoma
Carcinoma
ICPN
• low-grade
• high-grade
• biliary
• gastric
• intestinal
• oncocytic
PGA
• low-grade
• high-grade
Carcinoma
MCN
Carcinoma
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Normal epithelium
Metaplasia
Dysplasia
Low-grade BilIN
High-grade BilIN
Gallbladder
adenocarcinoma
25 µm
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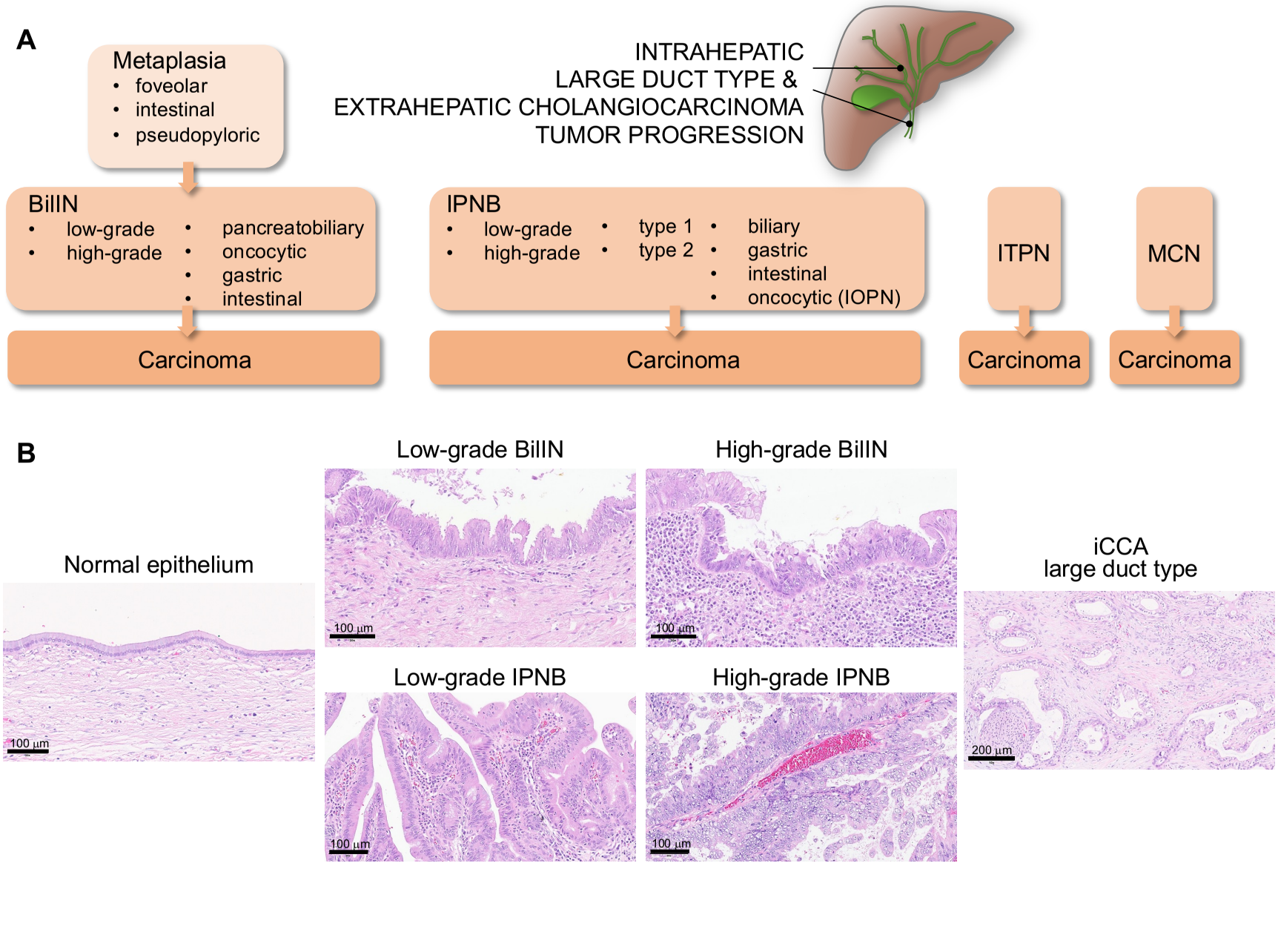
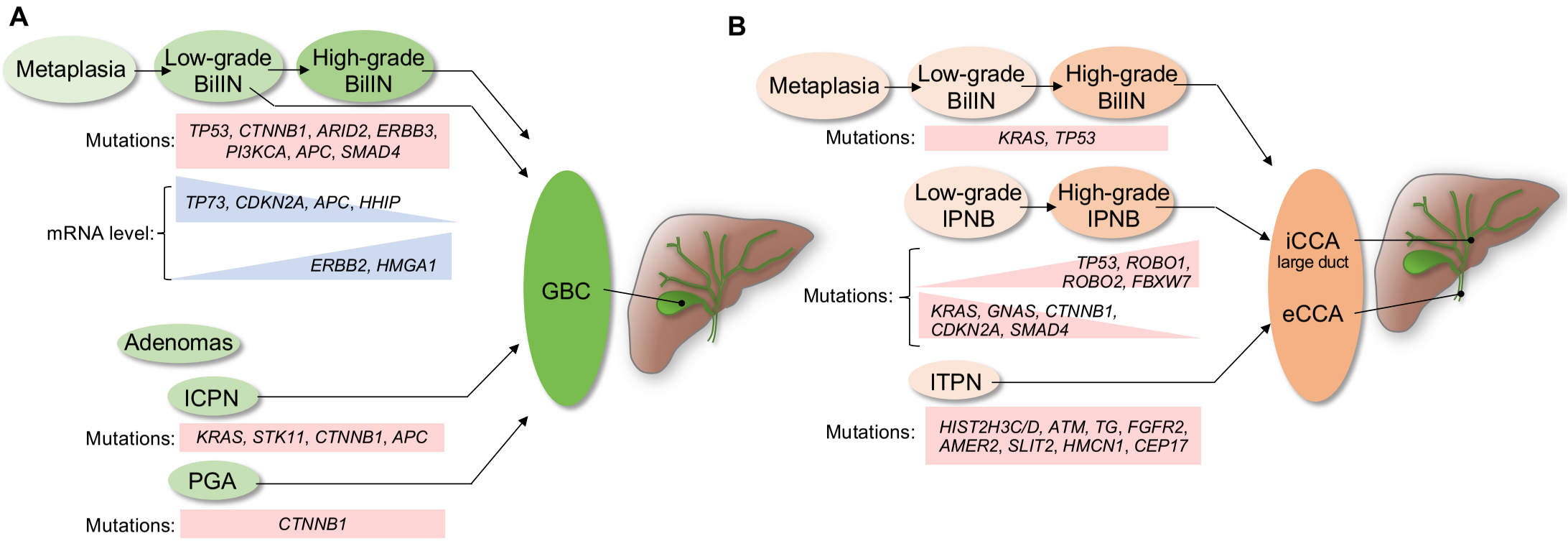

A
Metaplasia
• foveolar
• intestinal
• pseudopyloric
INTRAHEPATIC
LARGE DUCT TYPE &
EXTRAHEPATIC CHOLANGIOCARCINOMA
TUMOR PROGRESSION
BilIN
• low-grade
• high-grade
• pancreatobiliary
• oncocytic
• gastric
• intestinal
IPNB
• low-grade
• high-grade
• type 1
• type 2
• biliary
• gastric
• intestinal
• oncocytic (IOPN)
ITPN
MCN
Carcinoma
Carcinoma
Carcinoma
Carcinoma
B
Normal epithelium
Low-grade BilIN
High-grade BilIN
Low-grade IPNB
High-grade IPNB
iCCA
large duct type
100 µm
100 µm
100 µm
100 µm
200 µm


A
Metaplasia
Low-grade BilIN
High-grade BilIN
Mutations:
TP53, CTNNB1, ARID2, ERBB3, PI3KCA, APC, SMAD4
mRNA level:
TP73, CDKN2A, APC, HHIP
ERBB2, HMGA1
GBC
Adenomas
ICPN
Mutations:
KRAS, STK11, CTNNB1, APC
PGA
Mutations:
CTNNB1
B
Metaplasia
Low-grade BilIN
High-grade BilIN
Mutations:
KRAS, TP53
Low-grade IPNB
High-grade IPNB
Mutations:
TP53, ROBO1, ROBO2, FBXW7
KRAS, GNAS, CTNNB1, CDKN2A, SMAD4
ITPN
Mutations:
HIST2H3C/D, ATM, TG, FGFR2, AMER2, SLIT2, HMCN1, CEP17
iCCA
large duct
eCCA