# Pathology of the Bile Duct

Yasuni Nakanuma
*Editor*

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# Preface

About two to three decades ago, the diseases of the bile ducts, particularly the intrahepatic large bile duct and extrahepatic bile ducts, were mainly caused by infectious agents, gallstones, and malignancies, frequently associated with biliary obstruction, and these diseases were mainly dealt by surgeons therapeutically. However, in the recent two decades, the medical and pathological scopes of nonneoplastic and neoplastic diseases of these bile ducts have been much expanded and are now vigorously expanding. Particularly, imaging modalities and interventional and medical approaches to these biliary diseases have explosively advanced in these fields. In addition, with the emergence of several novel diseases such as IgG4-related sclerosing cholangitis and the discovery of preinvasive lesions of cholangiocarcinoma, new clinical and research approaches in biliary diseases have been explored. In addition, the discovery of peribiliary glands along the biliary tree and subsequent studies have caused diverse and exciting studies in this new field. Furthermore, several basic pathophysiologies of the biliary tree such as defense mechanisms, cellular senescence, innate immunity, and autophagy have been advanced and are actively being applied to the evaluation of the pathophysiologies of the bile ducts.

Accordingly, the roles of pathologists for diagnosis of these biliary diseases and for the evaluation of their pathogenesis have increased rapidly, and pathological knowledge and experiences are accumulating extensively. Many excellent clinical and basic research papers have been published and are being published in this field. So currently, the need for a comprehensive and concise pathology book covering recently accumulated pathological knowledge and experiences written in English is urgent and mandatory for pathologists and clinicians and basic scientists. There are a considerable number of excellent and concise pathology books of the liver and also of the pancreas available in bookshops, universities and hospital libraries, laboratories, and your room. However, there have been no pathology textbooks of bile ducts available at any place. At this most important and urgent time, *Pathology of the Bile Duct* has been just published.

This book is composed of 16 chapters, and these chapters are divided into two parts: basic understanding of bile duct pathology and practical understanding of biliary diseases. The former are composed of five chapters. In Chap. 1, recent

progress of bile duct embryology and anatomy including peribiliary glands is concisely described. This chapter is very important for understanding the other chapters. The biliary tree is constantly exposed to the bile containing many diverse and potentially toxic materials secreted from the hepatocytes and is also potentially exposed to the contents of the intestine which is continuous with the external world. So, the biliary tree is equipped with many elaborate defense mechanisms against toxic bile acids and other constituents and also possibly regurgitates hostile materials from the intestine. First, the bile ducts have a unique bicarbonate umbrella system against toxic bile acids and also an innate immune system to biliary constituents and also possibly regurgitate pathogens and antigenic materials. These defense issues are precisely described in Chaps. 2 and 3. Disturbances of these defense systems can also be involved in many biliary diseases. In Chap. 4, basic aspects of cell injuries of bile duct epithelia, particularly cellular senescence and cell death, are described, and the participation of the role of autophagy in these cell injuries is also referred. Autophagy is a topic of science at present, and in 2016, a Japanese researcher of autophagy, Dr. Osumi, got a Nobel Prize. In Chap. 5, the blood supply to the biliary system and its disturbance are described. The biliary tree is equipped with a characteristic blood supply, and this is a reason why several unique biliary diseases with ischemic change develop, and the pathologies of bile ducts and accompanying blood vessels are described in these diseases.

From Chaps. 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16, practical pathologies of biliary diseases are described. First, pathologies and pathogeneses of three representative immune-related biliary diseases such as primary biliary cholangitis, primary sclerosing cholangitis, and IgG4-related sclerosing cholangitis are described. These three types of biliary diseases have been much studied in these two decades, and particularly, IgG4-related sclerosing cholangitis is a newly discovered biliary disease. Primary biliary cholangitis has been extensively studied by immunopathologists, and Dr. Tsuneyama is one of the most diligent and productive persons in this field and has contributed much to the understanding of immune-mediated destruction of the bile duct in primary biliary cholangitis. The discovery of peribiliary glands along the biliary tree was a historic event, and subsequent studies have contributed much to the novel pathophysiological world of the biliary tree. The discovery of the relation between peribiliary cysts and peribiliary glands is one of the most fruitful products, and this field is described by referring to radiological findings in Chap. 9. Roles of innate immunity and viral infection have been reported to be important in the development of biliary atresia, and these are described with reference to peribiliary glands by Dr. Harada in Chap. 10. The role of epithelial-mesenchymal transition in the biliary fibrosis in biliary atresia is also explained with reference to the disordered innate biliary immunity.

From Chaps. 11, 12, 13, 14, 15 and 16, biliary neoplasms, which have been recently studied and discussed actively, are concisely and precisely described. Among them, cholangiocellular carcinoma is a difficult neoplasm which at one time belonged to intrahepatic cholangiocarcinoma and at other times belonged to combined hepatocellular cholangiocarcinoma. Many years ago, this carcinoma was an independent biliary neoplasm different from cholangiocarcinoma or hepatocellular

carcinoma. Dr. Kondo described its history and present and future in Chap. 11. So, readers will understand this puzzling neoplasm. Recent reclassification of cholangiocarcinoma into intrahepatic, perihilar, and distal cholangiocarcinoma is now accepted worldwide. However, it remains uncertain for many pathologists how to diagnose cholangiocarcinoma arising in the intrahepatic large bile ducts, and its relation to perihilar cholangiocarcinoma and peripheral intrahepatic cholangiocarcinoma is a dilemma. In Chap. 12, Dr. Aishima gave clear guidance for these questions which many pathologists have. Intraductal papillary neoplasm of bile duct, hepatic mucinous cystic neoplasm, biliary intraepithelial neoplasm, and intraductal papillary cystic neoplasm of the gallbladder and ampulla of Vater were introduced by the *2010 WHO Classification of Tumours of the Digestive System* as a preinvasive neoplastic lesion of the bile duct, gallbladder, and ampulla of Vater, and these preinvasive lesions are eventually followed by invasive carcinomas. These preinvasive neoplasms are conceptually very new and very important and also useful for practical clinical and pathological fields, and the pathologies of these preinvasive lesions are very clearly described in these four chapters. Particularly, recent clinical progresses make it possible to discover these preinvasive neoplastic lesions preoperatively. As the invasive biliary cancers are still an intractable malignant disease with high mortality, it is urgent to discover these neoplasms at the preinvasive stage, and in this context, these chapters are mandatory for clinicians and pathologists dealing with these malignant disorders.

Biliary pathophysiologies including pathologies of the bile ducts are one of the rapidly growing clinical and pathological and research fields, and we are delighted to compile a current collection of the knowledge and experience pertaining to this field in this book *Pathology of the Bile Duct*. We anticipate the next decade to be as exciting and productive as the past two decades. In this context, we hope this book may contribute much to the progress of biliary pathophysiology in the next decades.

Shizuoka, Japan Yasuni Nakanuma

# Acknowledgment

Although the limitation of space prevents us from thanking individually our collaborators and also other many pathologists, clinicians, and researchers who have contributed much in various ways to the progresses of the pathology of bile ducts, we would like to gratefully acknowledge the works of all these persons. Their discoveries, experiences, and practices are crystallized in this book *Pathology of the Bile Duct*. We also appreciate all editors who made possible the completion of the English version of this book. We strongly hope that this book will be useful and important and deliver numerous and latest informations on pathologies of bile ducts to the world.

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# Part 1
# Basic Understanding of Bile Duct Pathology

# Chapter 1
# Development and Anatomy of the Bile Duct

**Katsuhiko Enomoto and Yuji Nishikawa**

**Abstract** The bile duct system is a pathway of bile transportation from the liver to the intestine and plays a role of exocrine function of the liver. It consists of two different types of epithelial cells, hepatocytes and cholangiocytes. Anatomically as well as developmentally, the bile duct could be divided into intrahepatic bile duct (IHBD) and extrahepatic bile duct (EHBD; extrahepatic hepatic duct, gallbladder, cystic duct, and common bile duct) system. Initially, the secreted bile is transported through the apical side of hepatocytes called as bile canaliculus and then transferred to the duct system (IHBD and EHBD). EHBD characteristically develops the peribiliary glands (PBGs) which are suggested to be a niche of progenitor cell for the hepatobiliary system. Recent studies have revealed that development of IHBD and EHBD is differently regulated during developmental stage of the liver. EHBD arises from a part of the pancreatobiliary domain of foregut endodermal epithelium. In contrast, IHBD develops from the hepatoblasts inhabiting in the fetal liver as a common progenitor cell for hepatocytes and cholangiocytes. In this chapter, we first show histology of the bile duct system and review recent advances in the regulatory mechanisms of both IHBD and EHBD development.

**Keywords** Histology of the bile duct • Extrahepatic bile duct (EHBD) • Intrahepatic bile duct (IHBD) • Peribiliary gland (PBG) • Molecular regulation of the bile duct development

## 1.1 Introduction

The liver plays a pivotal role in the body's metabolic homeostasis by controlling various essential metabolic substances such as glucose, lipoproteins, etc., synthesizing important serum proteins, and detoxification of many xenobiotic materials. Bile

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secretion is also one of the most important functions of the liver. The bile duct system functions as a drainage path of the bile from the liver to the intestine. Anatomically, the bile duct could be divided into intrahepatic bile duct (IHBD) and extrahepatic bile duct (EHBD) [50]. Histologically, the bile duct system could be further divided into the bile canaliculus consisting of hepatocytes and the ductal structure constructed by cholangiocytes.

Major contents of the bile are bile acids, bilirubin, and cholesterol; those are synthesized in liver parenchymal cells, hepatocytes. Since there is no apparent duct system inside of the hepatic lobules, the secreted bile is transported through the bile canaliculus which is a structural conduit between two or three hepatocytes. At the margin of the portal area, the bile canaliculus and the bile ducts are connected via the canals of Hering (CoH) and the bile ductules which are small-sized bile ducts [51]. In the liver, interlobular bile ducts are interconnected and increase in their size and become hepatic ducts. The hepatic ducts join at the liver hilar region as a common hepatic duct; subsequently, after joining of the cystic duct, the duct is designated as the common bile duct and ends to the duodenum. The EHBD has long been thought as a rather simple duct system. However, it was demonstrated that the duct actually contains many accessory glands, named peribiliary glands (PBGs); those are located along with the duct system [42]. Recent studies highlight the function of PBGs as an additional niche of stem/progenitor cells for the hepatobiliary system [30].

The mouse liver development begins in the medial and lateral domains of the foregut endoderm at embryonic day (E) 8.5 of embryo, corresponding about 3 weeks of human embryo [74, 56]. The two domains migrate and become the hepatic diverticulum in the ventral foregut [69]. Two critical extracellular signaling molecules, fibroblast growth factor (FGF) from the cardiac mesoderm and bone morphogenetic protein (BMP) from the septum transversum mesenchyme (STM), induce invagination of hepatic diverticulum into STM [20, 25, 52]. Subsequently, hepatic diverticulum cells further migrate into STM with expression of the transcription factors Hex, Tbx3, and Prox1; those are related to induction of hepatic specification and cell proliferation. This migration needs vascular factors derived from mesenchymal blood vessels [36]. The hepatic specified cells are referred to as hepatoblasts and express hepatocyte-specific gene product, α-fetoprotein (AFP) and albumin (ALB), and at later stage, they give raise to hepatocytes and cholangiocytes.

The mechanisms of IHBD and EHBD development were extensively studied using the embryonic mouse model. IHBD formation occurs from hepatoblasts of the ductal plate, at relatively later stage (E15.5) of mouse development [32, 57, 7, 47–48, 76]. In contrast, EHBD originates from the pancreatobiliary domain of the ventral foregut at early stage of liver development. In association with migration of the hepatic diverticulum toward STM, a part of the pancreatobiliary domain gains biliary fate and subsequently forms gallbladder, cystic duct, and common bile duct [60].

In this chapter, we will show histology of the bile duct system and review recent progress in molecular regulation of the bile duct development.

## 1.2 Histology of the Bile Duct System

As the liver is a multifunctional organ, the majority of the membrane domain of hepatocytes (basolateral surface) faces the blood vessel, the sinusoid, to exchange a variety of metabolic substances and supply essential serum proteins, whereas the apical surface of hepatocyte at which the bile is secreted is a relatively limited (10–15% of the hepatocyte cell membrane) slit-like space and is called bile canaliculus (Fig. 1.1a). The bile canaliculus is sealed by the tight junction of adjacent hepatocytes to protect inflow of bile components into blood. The bile canaliculus membrane domain has many microvilli where a number of transport proteins localize for secreting bile components, bile acids, bilirubin, cholesterol, vitamins, heavy metal, and xenobiotics. The majority of these transport proteins belongs to the ATP-binding cassette (ABC) superfamily [3].

The apparent ductal structure consisting of cholangiocytes could be observed in the interlobular portal area. In the portal area, the basically three luminal structures, i.e., interlobular bile duct, portal vein, and hepatic artery, are seen in the fibrous connective tissue (Fig. 1.1b). The transitional structure between bile canaliculus and interlobular bile duct is known as the canals of Hering (CoH). It has been reported that both hepatocytes and cholangiocytes consist of the structural lining of CoH [51]; however,

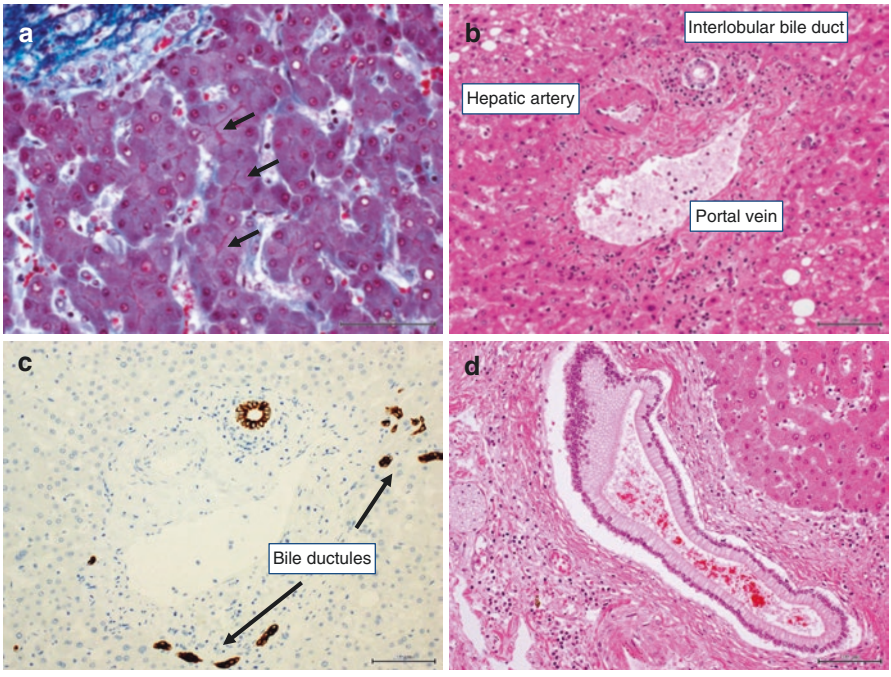


**Fig. 1.1** Histology of human bile duct. (**a**) *Arrows* show bile canaliculi between the adjacent 2–3 hepatocytes. (**b**) Histology of the portal area. The interlobular bile duct, portal vein, and hepatic artery are located in the fibrous connective tissue. (**c**) Immunohistochemistry for cytokeratin 19 indicates small bile ductules at the peripheral margin of the portal area. (**d**) A large hepatic duct consisting of high columnar cholangiocytes located at the hepatic hilum

the function and significance of CoH in the pathologic process is not fully determined. Recently, it has been shown that the numbers of CoH decrease in primary biliary cirrhosis (PBC) patients [28, 54]. Since pathogenesis of PBC is still unknown, further study is necessary whether CoH is a target or not in the PBC. At the downstream of the CoH, cytokeratin 19 (CK19)-positive bile ductules consisting of very small cuboidal cholangiocytes are seen at the peripheral zone of the portal area (Fig. 1.1c). The transitional area between hepatocytes and cholangiocytes may serve as a niche for stem/progenitor cells; those have a bipotential ability to differentiate toward hepatocytes and cholangiocytes under the normal and injured conditions in adult liver [39]. The cholangiocytes of intrahepatic bile duct vary in size and shape depending on their location. In the interlobular bile duct, cholangiocytes are small and cuboidal, whereas they become larger and columnar in shape along with the increase of duct size (Fig. 1.1d). Inside of the liver, bile ducts are designated as interlobular, septal, area, segmental, and hepatic ducts (>800 μm) according to their size [3, 21, 42]. Recent three-dimensional (3-D) analysis of the intrahepatic bile duct clearly showed that the cholangiocyte constitutes a finely organized duct network (biliary tree) in the whole liver [26]. They also showed a flexible remodeling of the biliary tree under the various liver injuries.

After the level of the hepatic ducts, i.e., the common hepatic ducts, the cystic duct and the common bile duct, they develop the peribiliary glands (PBGs), especially at branching points of the biliary tree [5–6, 8–9, 30, 42] (Fig. 1.2a).

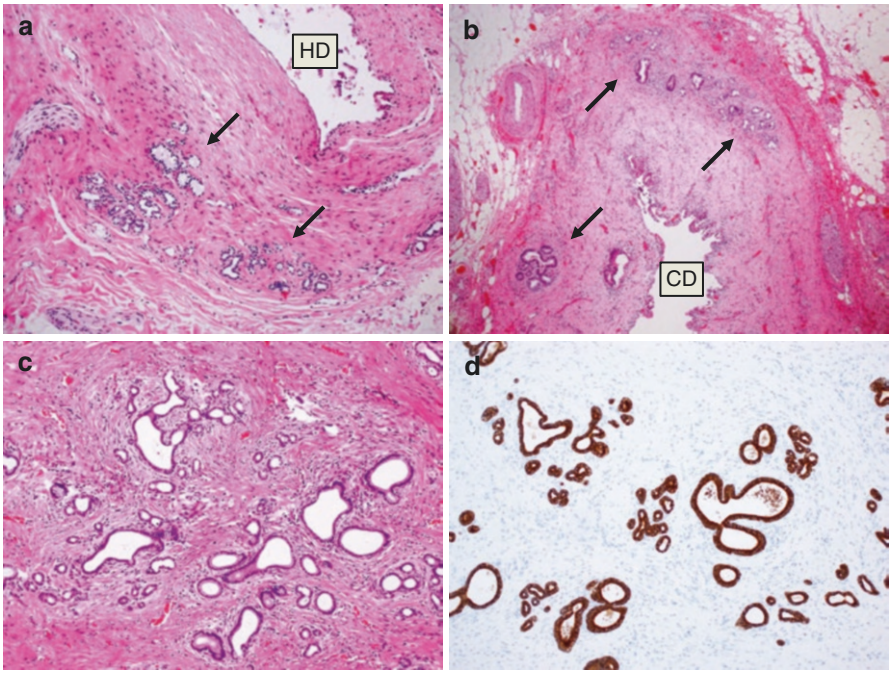


**Fig. 1.2** Peribiliary glands and the Luschka duct in the gallbladder. (**a**) Peribiliary glands (*arrows*) are a cluster of small ducts surrounding a large hepatic duct (HD) at the hepatic hilum. (**b**) The cystic duct (CD) has many peribiliary glands (*arrows*). (**c**) Luschka ducts located deep in the gallbladder wall consist of large and small ducts and are surrounded by fibrous connective tissue. (**d**) Luschka ducts are immunohistochemically positive for cytokeratin 19

Although the cystic duct has many PBGs (Fig. 1.2b), the gallbladder is known to have no or very few PBGs. In the serosal connective tissue of the gallbladder fossa, there is a special duct system, the so-called duct of Luschka, consisting of CK19-positive cholangiocytes (Fig. 1.2c). The duct does not belong to the gallbladder epithelium but is considered to be a branch of the right hepatic duct. Clinically, the duct may be associated with bile leakage after cholecystectomy.

Lanzoni et al. [30] showed that about 10% of PBGs in epithelium expresses the stem/progenitor cell markers (SOX17, SOX9, PDX1, EpCAM) and pluripotency markers (OCT4, SOX2, NANOG). These cells have a potential to give rise to cholangiocytes and surprisingly even hepatocytes and pancreas islet cells [5]. Obviously, further studies including the experimental model systems are needed to elucidate the significant role of PBGs in physiology and pathology of the EHBD.

## 1.3 Development of the Intrahepatic Bile Duct

It was shown that EHBD arises directly from the pancreatobiliary progenitor cells of the ventral foregut (describe later), whereas IHBD arises from hepatoblasts at the later stage of mouse embryo. The hepatoblasts are considered as bipotential progenitor cell being able to differentiate to either hepatocytes or cholangiocytes. In fact, it was clearly demonstrated that a single hepatoblast, isolated by flow cytometry according to the cell surface markers from 13.5E mouse liver, could differentiate into hepatocytes and cholangiocytes in vitro and in vivo [62]. The hepatoblasts in the embryonic liver are reported to express the stage-specific various characteristic markers, such as α-fetoprotein (AFP), albumin (ALB), EpCAM, Dlk1, etc. [19, 39].

During progression of embryonic liver maturation, cells committed to the biliary lineage (progenitor of the cholangiocyte) emerge in hepatoblast population located around the portal area. The excellent review articles described the molecular regulation of the biliary lineage differentiation from hepatoblasts and the tubulogenesis of committed biliary lineage cells [32, 47–48, 76].

In human embryonic liver, hepatoblasts with strong immunoreactivity for cytokeratins 8, 18, and 19 appeared around the mesenchyme of the large hilar portal vein at eighth week of gestation [50]. These hepatoblasts surrounding portal veins are called the “ductal plate.” In the part of the bilayered ductal plate, the tubular structure formation becomes visible at around 12 weeks of gestation. Then, tubular structures are incorporated into the portal mesenchyme, and this tubulogenic remodeling progresses from the hilar region to periphery of the liver.

In the mouse fetal liver, three-dimensional (3-D) morphological analyses of IHBD development demonstrated that the initial luminal cyst/segments appear at E13.5–E15.5, subsequently branching hierarchical luminal network appears at E18, and eventually IHBD maturation completes by 1 week after birth [64, 67]. Interestingly, it is suggested that bile secretion by hepatocytes may promote biliary tree network formation. These studies provide a new perspective in considering the regulation of maturation step in the whole organ level.

Studies of Alagille syndrome, which shows a variety of organ abnormalities including paucity of IHBD, demonstrated that mutations of the Notch signaling

genes, *jagged 1* and *notch2*, are crucial to induce the disease [33, 43, 38]. Thus, it seems likely that the Notch signaling is indispensable for the human IHBD development. But other molecules related to the biliary lineage commitment, proliferation, and tubulogenesis of human IHBD are not yet fully studied.

On the contrary, the gene-targeting mouse experiments revealed that a variety of molecules are involved in the embryonic development of mouse IHBD. Regarding regulation of IHBD development, three signaling pathways, Notch pathway, TGF-β pathway, and Wnt/β-catenin pathway, were extensively studied. In addition to the extracellular signaling pathway, several transcription factors which regulate the biliary commitment and tubulogenesis of IHBD during embryonic liver development have been identified [32, 47–48, 76].

### *1.3.1 Notch Pathway*

As shown in human Alagille syndrome, importance of the Notch signaling for IHBD development was confirmed in the mouse experimental model [37]. Since the Notch signal is mediated by cell-cell contact of Notch receptor 2 (NotR2) and its ligand Jagged 1 (Jag1)-expressing cells, it is necessary that cells expressing each molecules should be closely localized. In fact, Jag1 molecule was expressed in the portal mesenchymal cells as well as the endothelial cells of portal vein and hepatic artery, and NotR2 expression was detected in the bile duct cells but not in the ductal plate [29, 66].

Regarding Jag1, it was recently shown that a specific deletion of Jag1 in portal mesenchymal cells but not in endothelial cells failed tubular morphogenesis in embryonic liver [22].

The crucial role of Notch2 signaling was confirmed in mouse models by the experiment of functional loss and gain of its signaling cascade molecules. Hepatic double knockout of Notch1 and Notch2 and single Notch2 knockout showed an irregular ductal plate structure and disordered bile ducts, suggesting an indispensable role of Notch2 in IHBD development [18]. Upon Jag1 binding to Notch, the intracytoplasmic domain of Notch (Notch ICD) translocates to the nucleus and binds with recombination signal-binding protein immunoglobulin kappa J (RBP-jκ). This Notch ICD transcriptional complex activates gene transcription. Deletion of RBP-jκ led to a decreased number of Sox9-positive cells in E16.5 liver as well as downregulation of HNF1-β [72, 75]. This indicates that Notch is an essential signal receptor for the biliary lineage differentiation. Indeed, conditional overexpression of the intracytoplasmic domain of Notch2 (Notch2 ICD) in the liver resulted in acceleration, biliary fate differentiation, and induction of tubule formation in embryonic as well as adult liver [24, 68, 75]. More recently, hepatoblast-specific deletion of Notch2 (*Notch2$^{fl/fl}$/Alfp-Cre*) showed the complete defect of bile duct in embryonic and perinatal mouse liver; however, interestingly the disorganized secondary bile duct formation was observed in the survived mice after weaning [16]. The results may suggest the Notch signal-independent (possibly

ductal plate independent) bile duct formation mechanism potentially presenting in the liver. The recent data from our laboratory also showed that hepatocytes are able to transdifferentiate into cholangiocytes under the specific microenvironmental conditions in vitro and in vivo [41, 59].

### *1.3.2 TGF-β Pathway*

TGF-β signaling was shown to play a role in the biliary lineage differentiation of hepatoblasts. TGF-β signal reporter gene assay showed that TGF-β signal is strong around the portal vein in the control E12.5 liver, whereas there is no more such zone-restricted TGF-β signal in the HNF6/Onecut-2 knockout mice in which biliary differentiation is blocked [11]. A direct effect of TGF-β for inducing biliary differentiation using an ex vivo culture of embryonic liver was further demonstrated [11]. These indicate that transcription factor HNF6- and Onecut-2-dependent TGFβ signaling is necessary for biliary differentiation in the ductal plate hepatoblasts. In the E15.5 mouse liver, TGF-β-2 and TGF-β-3 were highly expressed in the periportal mesenchyme, and TGF-β receptor II was expressed in parenchymal side hepatoblasts of the asymmetric ductal plate tubules [1]. This suggests that the receptor-mediated TGF-β signal may promote biliary commitment in the receptor-expressed hepatoblasts. Smad4, a downstream signaling molecule of TGF-β, is suggested to be involved in the biliary differentiation process [49]. Recent study showed that the Hippo pathway is also involved in the biliary commitment of hepatoblasts [31]. YAP/TAZ activation by *Lats1/2* deletion leads to the proliferation of cholangiocytes by upregulation of TGF-β signaling.

### *1.3.3 Wnt/β-Catenin Pathway*

Wnt/β-catenin pathway is likely to be an additional important signaling for liver development in general [40]. The mouse embryonic hepatoblasts harboring β-catenin exon deletion exhibited a large decrease in the number of epithelial cells including hepatoblasts, hepatocytes, and cholangiocytes [65]. On the other hand, the mouse with β-catenin activation in hepatoblast caused by APC deletion (Alft-Cre x Apc $^{lox/lox}$) led to blocking of hepatocyte differentiation, instead of promoting biliary differentiation [14]. However, the most recent study which carefully revalidated the previous experiments using the Sox9-CreER mouse model concluded that β-catenin is dispensable for differentiation of hepatoblasts to cholangiocyte precursors. But this study also showed that β-catenin is necessary for maturation of biliary precursor cells in a time-restricted manner and subsequent bile duct formation [13]. Therefore, the further detailed regulatory mechanism of Wnt/β-catenin signaling on the maturation stage of IHBD needs to be clarified.

### *1.3.4 Transcriptional Regulation of IHBD Development*

Biliary lineage differentiation from hepatoblasts to cholangiocytes is necessarily required for gene expression of the biliary phenotype. Therefore, it is important to investigate the transcription factor network which regulates the biliary fate determination. Analyses of transcription factor-deficient mice revealed several transcription factors controlling differentiation and morphogenesis of IHBD.

HNF6 (Onecut-1) is the first reported transcription factor involved in IHBD development [10]. HNF6 lacking mice showed an incomplete biliary differentiation of the hepatoblasts and an abnormal morphology (cystic change) of IHBD. HNF6 also blocked the gallbladder development [10]. Furthermore, the deficiency of transcription factors, HNF1β, Onecut-2, or Hhex, induced disruption of IHBD development; thus, these are essential for normal IHBD development [11–12, 23]. As previously described, HNF6 and Onecut-2 function for keeping TGFβ signaling gradient (high in the periportal area and low in the parenchymal area) in developing liver and consequently define the periportal tubulogenesis of IHBD [11].

In normal mouse liver development, the suppression of the CCAAT/enhancer-binding protein α (C/EBPα, the basic leucine-zipper transcription factor) was observed around periportal hepatoblasts, suggesting that the loss of C/EBPα blocks hepatocyte differentiation and enhances IHBD development [58]. Indeed, the C/EBPα knockout liver exhibited multiple bile duct-like glandular structures expressing HNF6 and HNF1β mRNA [73]. Tbx3, a member of the T-box family, was shown to regulate proliferation of hepatoblasts and differentiation of hepatocytes; thus, the loss of Tbx3 function resulted in the biliary differentiation [63, 35]). Similar abnormal morphological phenotype was reported in the Prox1-deleted liver, and Prox1 is indicated by the downstream factor of Tbx3 [55]. Sall4 (a zinc finger transcription factor) overexpression experiment in the hepatoblasts by the virus-mediated gene transfer demonstrated the suppression of hepatocyte differentiation and the induction of biliary differentiation [45]. Collectively, these experiments seem to suggest that the abnormal bile duct formation is caused by block of the hepatocyte differentiation.

Antoniou et al. [1] have identified the SRY-related HMG box transcription factor 9 (Sox9) as an early biliary lineage marker. The Sox9-positive cells first appeared in the portal side of the primitive tubular structure (asymmetric tubule) derived from ductal plate cells at E15.5 embryo, whereas the parenchymal side cells still expressed the hepatoblast marker HNF4. The complete ductal structures in which all constituted cells showed positive nuclear Sox9 and membranous E-cadherin were seen at E18.5 mouse liver. This tubule formation in the ductal plate occurs from the hilum toward the periphery of the liver along with the portal vein axis. Since the bile duct maturation was retarded in Sox9-deficient mice liver, the role of Sox9 in IHBD development is suggested as a control of maturation step of primitive asymmetric tubule. In addition to Sox9, the Sox4 knockout and the Sox9/Sox4 double knockout

mouse liver also showed impairment of bile duct maturation with perturbation of the phenotypic differentiation of cholangiocytes [46]. Thus, it was suggested that the transcription factors Sox9 and Sox4 cooperatively promote bile duct maturation. The forkhead box transcription factor Foxa1/2 deletion in fetal mouse liver resulted in abnormal bile duct hyperplasia and fibrosis. This excess cholangiocyte proliferation is due to activation of IL-6, which is a growth-stimulating signal for cholangiocytes, and is usually negatively regulated by Foxa1/2 [34].

The spatiotemporal regulation and interaction of each transcriptional factor on the lineage switching in hepatoblasts may be a dynamic and complex process (Fig. 1.3). The distinct regulatory mechanism of biliary differentiation and morphogenesis remains to be elucidated.

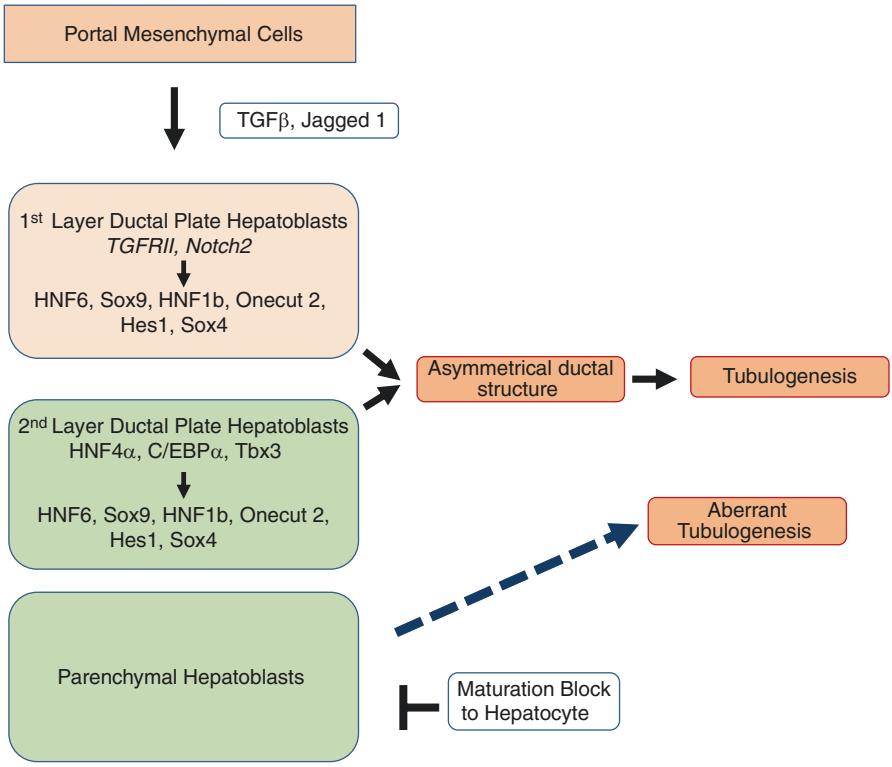


**Fig. 1.3** Development of intrahepatic bile duct. Specification of the ductal plate hepatoblasts to cholangiocytes is induced by TGF-β and Jagged 1 (ligand for Notch signaling) produced by periportal mesenchymal cells. Specification to cholangiocytes is associated with changes in the expression profile of transcription factors in the first layer of ductal plate hepatoblasts and followed by the second-layer hepatoblasts. The mechanisms of tubulogenesis are poorly understood. The blockage of hepatocyte maturation in the embryonic liver leads to aberrant duct formation

## 1.4 Development of the Extrahepatic Bile Duct

The development of EHBD is closely related to the stage of specification and migration of the hepatic and pancreatobiliary domain of the ventral foregut endoderm. The onset of hepatic specification occurs in the medial and lateral domains of the foregut endoderm at E8.5 of mouse embryo, as mentioned earlier. FGF signal from the cardiac mesenchyme and the following MAPK pathway activation are required for hepatic specification of domain cells [4]. At the same time period, the pancreatobiliary specification also occurs at the adjacent caudal part of the liver domain by positional escaping from the influence of FGF [2]. The so-called hepatic diverticulum is now considered to contain both hepatic and pancreatobiliary progenitor population, each located closely [15]. The hepatic progenitor cells (hepatoblasts) express transcription factors HNF4α and Hex and specific proteins ALB and AFP. The pancreatobiliary progenitor cells express *pancreatic and duodenal homeobox gene-1* (*pdx-1*) and *pancreas transcription factor 1* (*ptf1*), and targeting of the genes resulted in pancreas agenesis [27, 44]. For proper EHBD development, the transcription factors, HNF6, HNF1β, and Hex, are reported to be important. The transcription factor HNF6-deficient mice showed agenesis of the gallbladder and abnormal morphogenesis of EHBD [10]. In addition, knockout of Notch effector gene *hairy and enhancer of split-1* (*Hes-1*) resulted in defect of gallbladder, hypoplasia of EHBD, and appearance of ectopic pancreas tissue [17, 61]. The transcription factor Hes-1 is likely to determine the biliary fate of the pancreatobiliary progenitor cells.

Spence et al. [60] clearly showed the important role of SOX17 in the segregation of EHBD and pancreas phenotype in the pancreatobiliary progenitor cells. In E8.5 mouse SOX17 and PDX1 were co-expressed in the pancreatobiliary progenitor cells. However, the embryo of *Sox17* deleted in the ventral foregut showed loss of gallbladder and appearance of ectopic pancreas tissue at the area where common bile duct should be normally formed. In contrast, the embryo in which SOX17 was overexpressed in the pancreatobiliary progenitor cell resulted in the suppression of pancreas and appearance of ectopic biliary-like ducts. In sufficient SOX17 expression in the gallbladder and bile duct was reported to be associated with the phenotype of biliary atresia [70–71]. Therefore, it becomes now clear that expression of SOX17 in the pancreatobiliary progenitor cells determines the fate of EHBD (Fig. 1.4b). Furthermore, it was shown that SMT-derived BMP4 controls expression of SOX17 [53].

The anatomy and development of the boundary between IHBD and EHBD have yet been clearly demonstrated. To visualize the boundary, we have performed lineage tracing experiments for ALB (+) hepatoblast using *alb* $^{cre/+}$ × *Rosa26R* mice. In adult mice, all hepatocytes and intrahepatic bile duct cells are positive for β - galactosidase, whereas the gallbladder and common bile duct cells are negative. We have identfied the boundary of β -galactosidase-positive and - negative cells is the mid-portion of the extrahepatic hepatic ducts (Fig. 1.4a). Currently, we are investigating detailed developmental processes of the boundary between the two biliary systems (Nishikawa et al., manuscript in preparation).

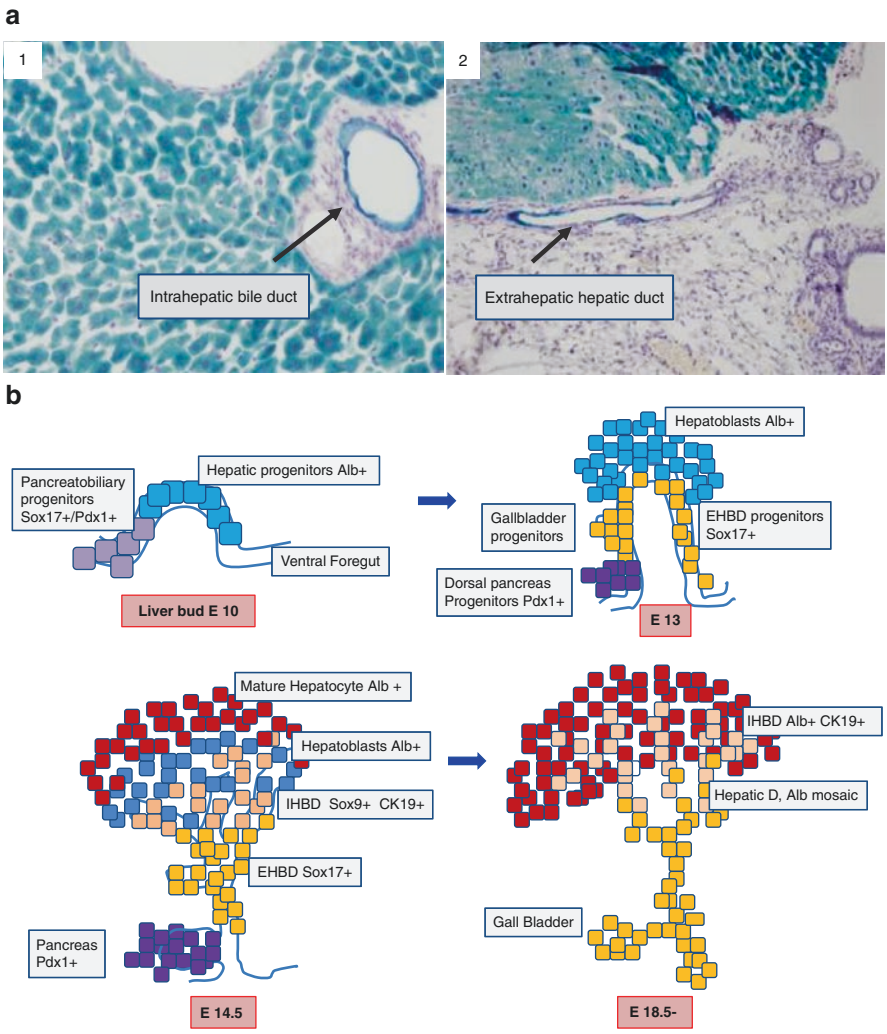


**Fig. 1.4** (**a**) Lac-Z staining of adult *alb*$^{cre/+}$ × *Rosa 26R* mouse liver. Hepatocytes and cholangiocytes of interlobular bile duct show positive staining for Lac-Z (1). Extrahepatic hepatic duct shows mosaic staining for Lac-Z (2). (**b**) Development of extrahepatic and intrahepatic bile duct system during embryonic stage is illustrated

## 1.5 Conclusion

Studies in the last decade have identified many molecules associated with the development of the bile duct system using advanced genetic technologies, such as conditional deletion of the particular genes and genetic cell fate tracing. The molecules of the specified signaling pathway and many types of transcription factors are

cooperated as regulators in the proper development of EHBD and IHBD. In the process of both EHBD and IHBD development, importance of the extracellular signaling mainly from mesenchymal cells and the subsequent intracellular transcription network becomes more evident. Although the precise molecular mechanisms, especially biliary cell differentiation from the pancreatobiliary domain of the foregut endoderm (EHBD development) and also biliary lineage segregation and morphogenesis from the hepatoblasts (IHBD development), are need to be determined, the accumulated evidence is now considerably useful for analysis of various types of human bile duct disorders. In fact, molecular analysis of human congenital bile duct malformation was performed [47–48]. In addition, the knowledge obtained through the bile duct development studies has helped understanding of the nature and cause of ductular reaction, which is often seen in injurious liver diseases, fulminant hapatitis and cirrhosis. The ductular reaction seems to be caused by the block of normal hepatocyte differentiation, because genetic ablation of transcription factors crucial for maturation programing toward hepatocytes in the embryonic mouse liver commonly resulted in a remarkable abnormal duct formation.

The normal liver development requires coordinated development of both the epithelial systems (cholangiocytes and hepatocytes) and the mesenchymal system (vasculatures and hematopoietic cells). Whole regulatory mechanisms of liver organogenesis may be more complex than described here, but obviously the further molecular analysis of liver organogenesis must contribute to the understanding of liver pathology.

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# Chapter 2
# Bicarbonate Umbrella and Its Distribution of the Bile Duct

**Shinji Shimoda**

**Abstract** Bicarbonate umbrella is a cell protective function from cytotoxic molecules by extracellular secretion of bicarbonate. Small bile ducts use this function to protect from hydrophobic bile acids, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs). On the other hand, primary biliary cholangitis (PBC) whose head of issue is characterized by the destruction of small bile ducts with the dysfunction of the bicarbonate umbrella as $Cl^-/HCO3^-$ exchanger anion exchanger 2 (AE2) activation. PBC has two features; an organ-specific autoimmune disease and a cytopathic biliary disease due to bile exposure that has strong cytotoxicity. Thus, bile ducts of PBC are exposed to two types of bile acids; hydrophilic and cytoprotective ursodeoxycholic acid (UDCA) and hyrophobic and cytotoxic bile acids. UDCA is effective to protect bile exposure in the early phase of the disease, and UDCA is not so effective in the advanced phase where abnormal acquired immunity has central roles for destruction of the small bile ducts.

**Keywords** Bicarbonate umbrella

## List of Abbreviations

| | |
|---|---|
| AE2 | Anion exchanger 2 |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| DAMPs | Damage-associated molecular patterns |
| FDA | Food and Drug Administration |
| FXR | Farnesoid X receptor |
| InsP3 | Inositol trisphosphate |
| PAMPs | Pathogen-associated molecular patterns |
| PBC | Primary biliary cholangitis |
| UDCA | Ursodeoxycholic acid |

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## 2.1 Introduction

Bile acid is synthesized as primary bile acid in the hepatic cells and secreted into the duodenum via the bile duct, after which it is transformed into secondary bile acid by the intestinal mycobacterial flora. Subsequently, most of the bile acid is reabsorbed during enterohepatic circulation, and the remainder is excreted. Since bile acid forms micelles and absorbs cholesterol into the bile, its role as a digestive enzyme, i.e., for lipid digestion and absorption in the small intestine, has attracted attention at first. Recently, it was revealed that bile acid has various functions. Bile acid functions on the nuclear receptor farnesoid X receptor (FXR), which is widely distributed throughout the body and controls bile synthesis and absorption by epigenetic functions [1]. Moreover, bile acid functions systemically via the cell membrane receptor TGR5 (also known as G protein-coupled bile acid receptor 1, G protein-binding receptor) to enhance energy metabolism [2]. While studies on bile acid function have been carried out, at the same time, bile acid studies focusing on the action thereof on secreted local biliary cells have also been carried out, through which the concept of a bicarbonate umbrella has been proposed.

## 2.2 What Is a Bicarbonate Umbrella?

A bicarbonate umbrella refers to the role of biliary cells which secrete bicarbonate and provide protection to cells from molecules with cytotoxic action. The term "bicarbonate umbrella" has been used since around 2010 because it acts in the same manner as an umbrella protects humans from raindrops [3]. The function of bicarbonate to protect the cell surface was revealed by the fact that bicarbonate secreted from gastric mucosa epithelium provides protection against gastric mucosa epithelial damage from gastric acid by creating a pH gradient [4]. Unlike the gastric mucosa, gallbladder, or large bile duct, the small bile duct has no mucin secretory ability; therefore, this bicarbonate secretion is the most important protective function from cellular damage in the small bile duct.

The exchange process of intracellular bicarbonate ions and extracellular chloride ions in the biliary cells is graphically indicated in Fig. 2.1 [5]. On the basement membrane side of the biliary cells, cAMP is produced within the cells by secretin stimulation. Chloride ions are released onto the extracellular luminal side via cAMP-sensitive chloride ion channels (cystic fibrosis transmembrane conductance regulator [CFTR]), releasing ATP at the same time. The released ATP enhances inositol trisphosphate (InsP3) production within the cell via P2Y. InsP3 is also produced via muscarine receptors on the basement membrane side. Moreover, oxidant stress controls InsP3 expression by inducing transcription factors [6]. InsP3 acts on the endoplasmic reticulum calcium channels, releasing calcium ions of the endoplasmic reticulum into the cells. Calcium ions release chloride ions extracellularly via calcium ion-dependent chloride ion channels (CaCl) at the luminal side cell

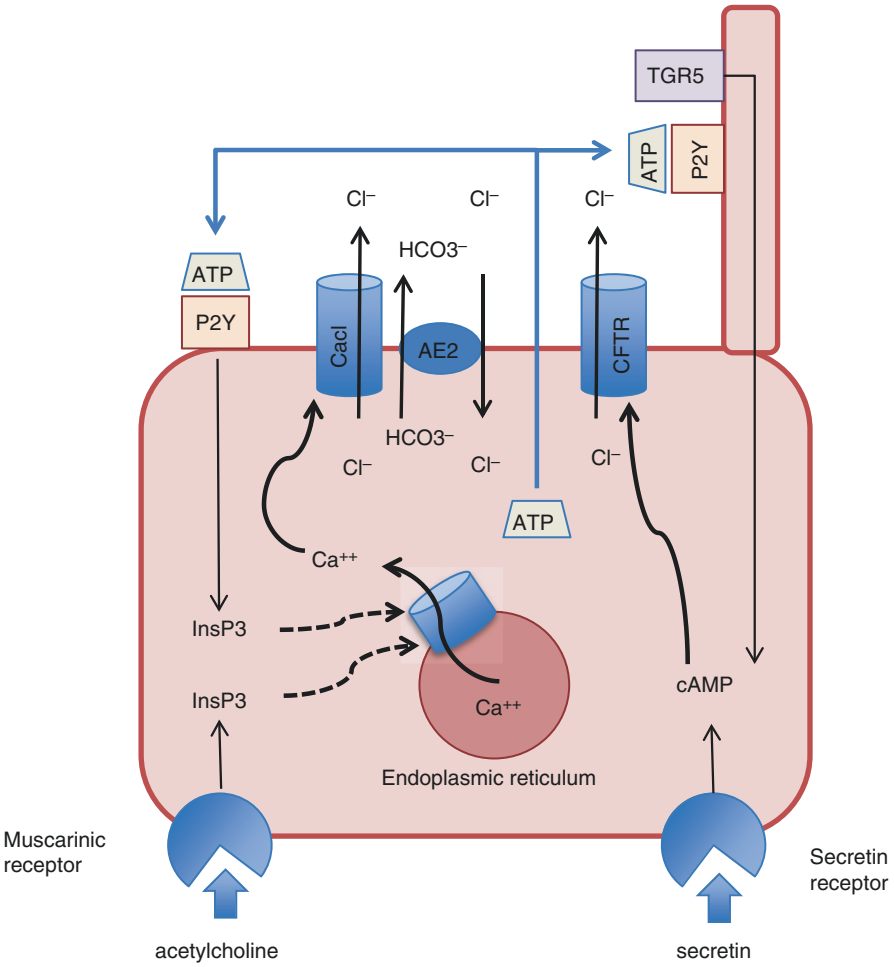


**Fig. 2.1** The biliary bicarbonate umbrella. A number of signaling pathways regulate the formation of the biliary bicarbonate umbrella. Biliary epithelial cells are responsible for secretion of bicarbonate into bile ducts. Calcium ion signal plays an important role in the regulation of secretion via basolateral muscarinic receptor. And stimulation of secretin receptors results in increased cAMP that induces release of ATP into bile ducts. This stimulates apical P2Y, which promotes InsP3 production and calcium ion release. AE2 is the chloride ion/bicarbonate ion exchanger

membrane. Extracellularly released chloride ions are exchanged with bicarbonate ions in the cells through anion exchanger 2 (AE2), excreting bicarbonate ions to the extracellular luminal side. In this way, by taking advantage of secretin and muscarine stimulation from the basement membrane, intracellular calcium signals, and AE2 at the lumen, bicarbonate ions cover the cellular lumen as an "umbrella," protecting the cells from substances with damaging action.

Furthermore, cilium, which extends to the luminal side of the biliary cells, functions as a bile osmotic sensor, controlling intracellular calcium ion exchange, ATP

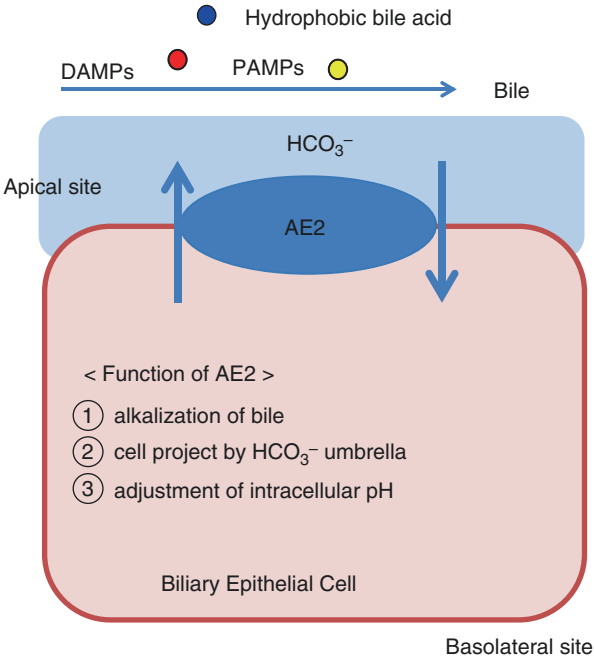


**Fig. 2.2** Bicarbonate umbrella protects harmful stimulations such as hydrophobic bile acids, DAMPs, and PAMPs from the bile duct (apical site) by AE2

release, and bicarbonate secretion. cAMP is intracellularly produced by bile stimulation, via ciliary G protein-coupled bile acid receptor 1 (also known as TGR5). cAMP plays a role in releasing chlorine ions extracellularly at the lumen via chlorine ion channels, thereby controlling bicarbonate ion secretion. In addition, biliary cells produce several neurosecretory substances including melatonin, thereby controlling functions such as proliferation and bicarbonate secretion [7]. Bicarbonate ions secreted to the luminal side maintain the cell surface as a weak alkali, protecting biliary cells from cellular damage caused by hydrophobic bile acid flowing along the luminal side (Fig. 2.2).

## 2.3 Abnormal Bicarbonate Umbrella and Biliary Disorders

It has been reported that in biliary cells with primary biliary cholangitis (PBC), AE2 activation is deteriorated and AE2 activation cannot be recovered by cAMP/ATP stimulation [8]. In an evaluation using human biliary cell culture lines, it was revealed that the extracellular pH and the degree of AE2 expression in the biliary cell determine biliary cellular damage [9]. Moreover, due to the finding that in addition to the increase in ALP and IgM, anti-mitochondrial antibodies defining the disease specificity of PBC are expressed in AE2 knockout mice, or

the finding that inflammation of the portal area is expressed, which is characterized by $CD8^+$ T cell/$CD4^+$ T cell/B cell invasion into the vicinity of damaged bile ducts, it was determined that AE2 knockout mice act as a PBC model [10]. In addition, through a genetic search, AE2 and TNF-α were extracted as factors defining ursodeoxycholic acid (UDCA) treatment reactivity [11]. Based on these findings, PBC is believed to be caused by the suppression of bicarbonate ion release into the bile due to damaged AE2 activity. The possibility of epigenetic gene expression control by microRNA has also been discussed, revealing that enhanced expression of microRNA 506 in the PBC biliary cells controls AE2 expression [12].

It was identified that regarding PBC, AE2 expression in the lymphocytes decreases [13], and specifically in $CD8^+$ T cells, AE2 is involved in the immune function thereof [14]; therefore, in the future, analyses taking AE2 function regarding immune cell abnormality into consideration, as well as biliary cell abnormalities, are required when analyzing PBC.

UDCA is the only therapeutic agent for PBC currently approved by the Food and Drug Administration (FDA) which improves not only laboratory data such as ALP, AST/ALT, and IgM but also hepatic tissues, leading to an improvement in prognoses by preventing them from becoming fibrotic [15, 16]. While the medical effect of UDCA has not been completely revealed, possible effects include improvement in immune modulation, cytoprotective action, and the bile acid pool [17]. UDCA acts to protect biliary cells from damage by inducing abundant bile acid from bicarbonate and creating a bicarbonate umbrella on the luminal side of the biliary cells [5]. Moreover, it was also revealed that by adding steroids to UDCA, AE2 expression in the liver is enhanced [18].

## 2.4 Conclusion

In the process of discovering the physiological function of the biliary cells, the important role of bicarbonate umbrellas as a natural defense mechanism of the biliary tract was indicated. Due to the facts that AE2 knockout mice can act as a PBC model and that in PBC, AE2 is largely involved mainly with the bile ducts, with the PBC therapeutic agent of UDCA also playing a role via AE2, the bicarbonate umbrella has become a necessary concept in PBC cholangiopathy. In the future, in order to control the action of PBC that is uncontrollable by UDCA alone, it is believed that treatments will be devised and drugs will be discovered with the objective of further enhancing the expression and function of AE2.

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# Chapter 3
# Innate Immunity of the Bile Duct and Its Disorder

**Atsumasa Komori**

**Abstract** The interaction between pattern-recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) is likely to provide first-line cell-autonomous innate immunity around the bile duct. Inflammatory cytokines and antimicrobial peptides are induced from biliary epithelial cells (BECs) after the interaction between PAMPs and PRRs prevents the overgrowth of pathogenic microbes. On the contrary, dysregulated and sustained activation of PRRs on BECs would in turn give rise to perpetual inflammation, possibly associated with the pathogenesis of chronic inflammatory diseases of the bile duct or "cholangiopathy."

The second-line innate immunity of the bile duct is governed by cellular innate immunity, including tissue-resident lymphocytes, such as natural killer (NK) cells and innate lymphocytes. Tissue-resident lymphocytes around the bile duct are expected not only to act as sentinel innate cellular players that respond to biliary infection in coordination with BECs but also to modulate and aggravate the fate of cholangiopathy. Antagonizing immunologically relevant molecules that are responsible for the recruitment of cellular innate immunity around the bile duct could be a breakthrough strategy for the treatment of intractable cholangiopathy in the future.

**Keywords** Pattern-recognition receptors (PRRs) • Pathogen-associated molecular patterns (PAMPs) • Biliary epithelial cells (BECs) • Cholangiopathy • Toll-like receptor (TLR)

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## Abbreviations

| | |
|---|---|
| APCs | Antigen-presenting cells |
| BECs | Biliary epithelial cells |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CHILPs | Common helper innate lymphocyte progenitor cells |
| CILPs | Common ILC precursors |
| CIS1 | Suppressor of cytokine signaling 1 |
| CLPs | Common lymphoid progenitors |
| CLRs | C-type lectin receptors |
| EHBD | Extrahepatic bile duct |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| HMGB1 | High-mobility group box 1 |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ILC | Innate lymphoid cells |
| iNKT | Invariant natural killer T |
| LPS | Lipopolysaccharide |
| lr-NK | Liver-resident NK |
| LTA | Lipoteichoic acid |
| MAIT | Mucosal-associated invariant T cell |
| MDA5 | Melanoma differentiation-associated protein 5 |
| MHC | Major histocompatibility |
| MR1 | MHC-related molecule-1 |
| Mx1 | Myxovirus resistance 1 |
| MyD | Myeloid differentiation factor |
| NK cells | Natural killer cells |
| NKPs | NK progenitor cells |
| NKT | Natural killer T cell |
| NLRs | Nucleotide-binding oligomerization domain-like receptors |
| PAMPs | Pathogen-associated molecular patterns |
| PBC | Primary biliary cholangitis |
| PRRs | Pattern-recognition receptors |
| PSC | Primary sclerosing cholangitis |
| QTLs | Quantitative trait genes |
| RIG1 | Retinoic acid-inducible gene-I |
| RLRs | Retinoic acid-inducible gene-like receptors |
| TGF | Transforming growth factor |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| $T_{RM}$ | Tissue-resident adaptive memory T cell |

## 3.1 Introduction

Chronic inflammatory diseases of the bile duct wherein biliary epithelial cells (BECs) or cholangiocytes are targeted for pathological cell damage, often leading to cholestatic liver failure, are commonly described with the term "cholangiopathy." Cholangiopathy can be roughly classified into autoimmune, hereditary, and infection-related types. In the two major forms of autoimmune cholangiopathy, that is, primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), the development of acquired autoimmunity against certain autoantigens expressed on BECs has been regarded as pathognomonic, because inflammatory effector cells around BECs exhibit T lymphocyte predominance. On the contrary, BECs themselves may not be an innocent "victim"; a decade of BEC research, even most of which was performed in vitro and ex vivo, has clearly demonstrated that pathogen-associated molecular patterns (PAMPs) are recognized by pattern-recognition receptors (PRRs) expressed on BECs, resulting in the modulation of the inflammatory milieu by induction of cytokine, chemokine, and co-stimulatory/inhibitory receptors [1]. Innate immunity by BECs, originally described as the physiological defense system in barrier sites, thus may play a substantial role also in cholangiopathy, while inborn myeloid differentiation factor (MyD)88 deficiency, which causes Toll-like receptor (TLR) signaling defects, is not apparently associated with the risk of recurrent cholangitis [2].

This chapter will define biliary innate immunity in a much broader sense, covering cellular innate immunity by tissue-resident lymphocytes, such as NK cells and innate lymphocytes. PAMP/PRR systems on BECs, as well as cellular innate immunity around the bile duct, may operate in line with innate-adaptive connections in autoimmune cholangiopathy, whereas their dysregulation could be the primary driver for infection-associated cholangiopathy.

## 3.2 Pattern-Recognition Receptors (PRRs) and Pathogen-Associated Molecular Patterns (PAMPs) Around the Bile Duct

Because antimicrobial peptides like β-defensin are constitutively released from BECs into bile juice, bacterial flora in the duodenum cannot enter the bile duct in principle, making the conduit sterile. However, trace amounts of PAMPs, lipopolysaccharide (LPS) from gram-negative bacteria, lipoteichoic acid (LTA) from gram-positive bacteria, and their nucleic acids, all present in the duodenum, may regurgitate back into the bile duct. It is possible that clearance of PAMPs would be compromised in cholangiopathy.

Sasatomi et al. first reported in an immunohistochemical study using anti-lipid A antibody that LPS, the TLR4 ligand, accumulated in damaged BECs in both PBC

and PSC [3]. Harada et al. further demonstrated by PCR analysis that the bacterial 16S ribosomal RNA gene derived from *Propionibacterium acnes* was amplified in the DNA extracted from the human PBC liver characterized with epithelioid granuloma [4]. Tsuneyama et al. also revealed LTA-engrafted monocytes around the damaged BECs of PBC [5]. Following these reports, the expression pattern of TLRs on BECs, coupled with the analysis of effector cytokines after TLR stimulation, has been studied extensively. This work has expanded according to the accumulating knowledge about other PAMPs, namely, TLR1, 2, 4, 5, and 6, and C-type lectin receptors (CLRs) on the cell surface respond to corresponding PAMPs, whereas TLR3, 7, and 9, retinoic acid-inducible gene-like receptors (RLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs) in the cytoplasm do so to their ligands [6]. Inflammatory cytokines and antimicrobial peptides which are subsequently released from BECs after the interaction between PAMPs and PRRs prevent the overgrowth of pathogenic microbes. Fine-tuning of the response to PAMPs through PRRs on BECs is of particular importance, because dysregulated and sustained activation of PRRs would likely give rise to perpetual inflammation.

Endotoxin tolerance, an unresponsiveness to LPS stimulation originally demonstrated in intestinal epithelial cells, may in part be responsible for such negative-feedback regulation. Harada et al. revealed that the second LPS stimulation to BECs was not transduced properly through TLR4, because its signaling pathway was suppressed by IRAK-M, the downstream kinase in TLR4, which was pre-activated by the initial LPS administration [7]. Tolerance to PAMPs, nevertheless, is not likely to be a universal phenomenon among distinct PRRs, because TLR3/retinoic acid-inducible gene-I (RIG1)/melanoma differentiation-associated protein 5 (MDA5) activation by poly dI-dC, which was monitored by the induction of effector molecules, such as interferon (IFN)-β, myxovirus resistance 1(Mx1), and TNF-related apoptosis-inducing ligand (TRAIL), was not inhibited by the pre-stimulation of the receptor [8]. The pathogenesis may thus hinge, at least partly, on biliary atresia, for which a causal association to rotavirus infection has been proposed; prolonged TLR3 activation in BECs without tolerance to poly dI-dC from the virus could be linked to the progression of disease through the perpetual release of effector cytokines.

Moreover, the presence of pathogenic microbes may affect the steady-state level of PRRs, as well as those of downstream signaling molecules, by posttranscriptional mechanisms. Expression of TLR4 was increased in the presence of *Cryptosporidium parvum* in BECs through the downregulation of *let-7*, a microRNA negatively regulating TLR4 abundance [9]. One suppressor of cytokine signaling (CIS1) has been shown to be a negative regulator for various cytokine signalings; its translation is suppressed by either miR-98 or *let-7*. LPS treatment or the infection of BECs by *Cryptosporidium parvum* inhibited the steady-state level of those miRNAs, resulting in the increase in CIS1 [10].

Aberrant tuning of the response to PAMPs through PRRs on BECs might also be postulated as a molecular pathogenesis in hereditary or autoimmune cholangiopathy. Loss of function in cystic fibrosis transmembrane conductance regulator

(CFTR) model mice effectively suppressed endotoxin tolerance, giving rise to sustained activation of TLR4 and of NF-kb signaling, suggesting the role of TLR4 as an aggravating factor in cystic fibrosis [11]. This is the case also in autoimmune cholangiopathy. Immunoglobulin (Ig)G purified from PSC sera stimulated BECs from human PSC liver in vitro, resulting in the upregulation of TLR4 and 9 through the activation of ERK1/2 signaling. Treatment of BECs with such IgG augmented the biological readout of subsequent LPS and CpG-DNA stimulation, that is, the release of interleukin (IL)-1β, IL-8, IFN-γ, tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)-β. The expression levels of TLR4 and 9 were indeed higher in PSC patients with anti-BEC antibodies compared to those without. Anti-BEC antibodies, which are known to be present in PSC at high frequencies, may exert their biological activity, at least in part, through an amplification of TLR signaling [12]. Abundantly expressed IFN-γ and TNF-α in the PSC liver were further postulated to be a driver for enhanced activation of TLR4, by inducing loss of LPS tolerance and TLR4 upregulation [13]. The interaction of PRRs and PAMPs in BECs in autoimmune cholangiopathy should be evaluated from the anatomical point of view in the future, that is, according to the relative distribution of membranous receptors, ligands, and modulating cytokines, between apical (duct luminal site) and basolateral surface (portal tract).

## 3.3 Activated BECs, Surrounding Stromal Cells, and Endogenous PAMPs in the Inflammatory Milieu: The Connection Between Cholangiopathy and Tissue Fibrosis

Once BECs become activated by inflammatory cytokines and microbial PAMPs that are present in the liver around the bile duct, they subsequently induce the expression of a myriad of inflammatory cytokines (e.g., IL-1, IL-6), chemokines (e.g., CCL2, CX3CL1), and fibrogenic growth factors (platelet-derived growth factor, TGF-β, connective tissue growth factor, endothelin-1), adding inflammatory complexity to fibrogenic potential; the biologically relevant targets of the above humoral factors released by BECs are stromal cells, consisting of peribiliary gland-associated fibroblasts, portal fibroblasts, stellate cells, and endothelial cells. After stromal cells have been stimulated by BECs, phenotypical transformation ensues, leading to the remodeling of the inflammatory tissue, by transcription and proteolysis, making the inflammatory microenvironment a rich source of bioactive endogenous TLRs [14] (e.g., biglycan, low-molecular-weight hyaluronate, heparan sulfate, and high-mobility group box 1 (HMGB1)) and of Wnt ligands. Consequently, an unrestrained, multicellular positive-feedback loop toward biliary fibrosis could be established around the bile duct.

## 3.4 Cellular Innate Immunity Around the Bile Duct: The Role of Tissue-Resident Innate, Innate-Like, and Adaptive Lymphocytes

Tissue-resident lymphocytes around the bile duct are likely to act not only as sentinel innate cellular players that respond in coordination to infection by BECs but also modulate the fate of inflammation in cholangiopathy.

Tissue-resident lymphocytes exhibit distinct ontogenies in nature [15]. Common lymphoid progenitors (CLPs) in the bone marrow differentiate into the precursors of T cells, natural killer (NK) cells, and innate lymphoid cells (ILCs). T cell precursors enter the thymus, where they develop into naïve T cells that harbor rearranged antigen receptors. Naïve T cells subsequently mature into two populations, according to whether they recognize antigens in a major histocompatibility (MHC)-restricted manner, that is, adaptive T cells and innate-like T cells. Innate-like T cells, commonly referred to as natural killer T (NKT) cells, express invariant T cell receptors and recognize antigens, including lipid antigens presented by CD1 molecules (Type I and II NKT) and small metabolites presented by the MHC-related molecule-1 (mucosal-associated invariant T cell, MAIT) [16].

In the bone marrow, common ILC precursors (CILPs) give rise to common helper innate lymphocyte progenitor cells (CHILPs) and NK progenitor cells (NKPs); innate lymphoid cells (ILC)1, 2, and 3 are derived from the former, whereas NK cells are differentiated from the latter (Fig. 3.1). Collectively, tissue-resident lymphocytes consist of ILCs, NK cells, unconventional T cells (NKTs, MAITs, and γδ T cells), and tissue-resident adaptive memory T ($T_{RM}$) cells [17]. Tissue-resident lymphocytes include sense microbial products, cytokines, alarmins, and stress ligands at the barrier surface, constituting as the source of antimicrobial effector molecules, such as IFN-γ, TNF-α, IL-17, as well as Il-4/5/13.

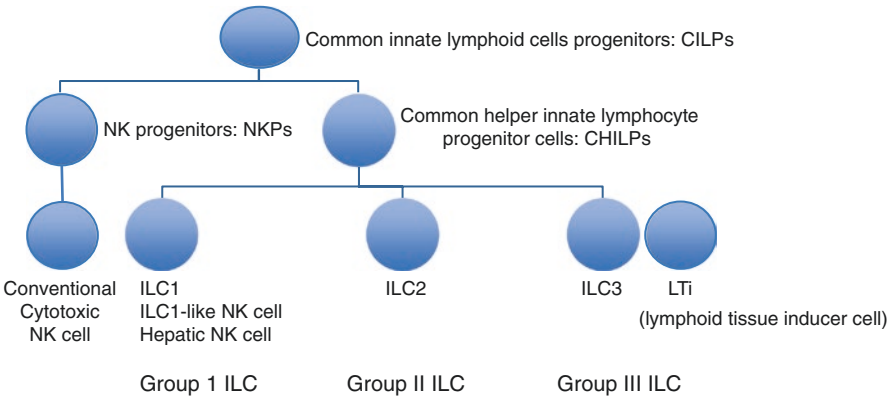


**Fig. 3.1** Innate lymphocyte

The primary barrier surface functions of resident lymphocytes in the liver parenchyma and the bile duct are likely to be distinct; since the former are routinely exposed to toxins and pathogens that reach the circulatory compartment from the gut, they may be regarded as tolerogenic to prevent undesirable inflammation. On the other hand, the latter surface, composed of the small intralobular bile duct with hepatocytes proxy to the large extrahepatic bile duct, may exhibit a more hierarchized nature in their resident lymphocytes. Therefore, the following observations that were mostly demonstrated in the liver-resident cells, as well as in the small intralobular bile duct, may not apply to the extrahepatic large bile duct; indeed, the entire immunological universe of bile duct-associated lymphocytes is still elusive.

Liver-derived lymphocytes have been known to be enriched in $CD56^{bright}$ NK cells and $CD161^{bright}$ MAIT cells in humans [16]. Jo et al. recently demonstrated that the TLR8 agonist ssRNA40 selectively activated human ex vivo liver-resident innate immune cells to produce a substantial amount of IFN-γ and that $CD56^{bright}$ NK cells and $CD161^{bright}$ MAIT cells were responsible for the production [18]. In terms of the mechanism, TLR8 has been shown to primarily target intrahepatic monocytes, inducing the release of innate cytokine IL-12 and IL-18 to in turn activate NK and MAIT cells.

### *3.4.1 NK Cells*

The number of NK cells around the portal tract of the human PBC liver was reported to be increased [19], either through clonal expansion of residual cells or by recruitment from systemic circulation. To characterize the relevance of such phenomenon, Shimoda et al. analyzed the relative contribution of NK cells in modulating autoreactive T cells in vitro [20]. Spleen-derived NK cells that were pre-stimulated with TLR3/TLR4 agonists produced IFN-γ after co-culture with BECs. The cell-number ratio of NK cells to autologous BECs was positively associated with the probability of BEC cell death in culture; cytotoxic NK cells are autoantigen independent in nature and subsequently gain the potential to stimulate the growth of autoreactive $CD4^+$ T cells in the presence of antigen-presenting cells (APCs). On the other hand, at a low NK/BEC ratio, IFN-γ production was sufficient to facilitate MHC class I and class II molecule expression on BECs but protected them from cell lysis by subsequent NK cell exposure, whereas autoreactive $CD4^+$ T cells became cytopathic to BECs. The spatiotemporal interaction between NK cells and BECs may determine the magnitude of $CD4^+$ T cell-mediated autoimmunity against BECs, though the direct interaction between innate/innate-like lymphocytes and $CD4^+$ T cells proximal to BECs should also be investigated as potential part of PBC pathogenesis (Table 3.1) [21].

Though Shimoda et al. utilized spleen-derived conventional NK cells in their ex vivo experiments, the liver-resident NK cells that are developed in the extramedullary space have recently gained more attention. Hudspeth et al. recently demonstrated that in healthy livers nearly 50 % of the entire liver NK cell population were $CD56^{bright}$ liver-resident NK cells (lr-NK) that were phenotypically and transcrip-

**Table 3.1** Induction, progression, and maintenance of primary biliary cholangitis: hypothetical requirement and sequel in pathogenesis

| |
|---|
| Requirement 1: Genetic background |
| Requirement 2: Environment/commensal and infectious microbes/PAMPs |
| *Stage 1: Inductive phase* |
| Step 1: Exogenous self-mimicry peptide-induced priming of autoreactive T and B cells that could respond to mitochondrial antigen |
| Step 2: Cell damage that enables to present mitochondrial antigen to autoreactive T and B cells |
| Step 3: Affinity maturation and clonal expansion of mitochondria antigen-reactive T and B cells |
| Step 4: Epitope spreading of mitochondria antigen-reactive T and B cells |
| *Stage 2: Effector phase* |
| Step 5: Injury of the intralobular bile duct-lining cholangiocytes caused by autoantigen-reactive cytotoxic effector cells |
| Step 6: Injury of cholangiocytes caused by innate lymphocytes (NK cells?) |
| Step 7: Adaptive-to-innate and innate-to-adaptive relay that amplify the injury of cholangiocytes |
| *Stage 3:* Damage of hepatocytes, fibrogenesis, and cholestasis |

tionally distinct from their counterparts in the peripheral blood [22]. $CD56^{bright}$ lr-NK resided in hepatic sinusoids by virtue of CD69, CCR5, and CXCR6 and co-localized with $CD56^{dim}$ conventional NK cells that were otherwise characterized by $CX_3CR1$, the homing receptors to peripheral tissues. Memory-like human $CD3^{-}CD49a^{+}CD56^{+}$NK cells that are the putative counterparts of those previously reported in mice [23] were also identified, albeit at low frequencies. At any rate, lr-NK cells are likely distinct cell populations from the ones that are amplified in number around the portal tract and characterized by $CX_3CR1$ expression. The sites of priming and activation of lr- or conventional NK cells, typically in the parenchyma or along the bile duct, are of particular importance for the contribution of NK cells to cholangiopathy. Namely, gut microbiota and enterohepatic correlation, in addition to microorganisms in the bile duct, should be taken into consideration when examining the role of NK cells in cholangiopathy.

Okamura et al. demonstrated that the number of $CX_3CR1^{+}$NK cells was increased in the livers of biliary atresia patients, accompanied by the augmented expression of CXCL1 [24]. Antagonizing $CX_3CR1$ or CXCL1 to prevent the recruitment of NK cells around the bile duct could be a future treatment strategy.

### *3.4.2 Unconventional T Cells*

MAIT cells comprise up to 45 % of liver T cells in humans, being predominant liver-resident lymphocytes, whereas invariant natural killer T (iNKT) cells represent no more than 1 % of the population. MAIT cells are characterized by the

expression of TCR Vα7.2-Jα33 and respond to vitamin B metabolites from pathogenic and/or commensal bacteria presented on the phylogenetically highly conserved MHC-related molecule-1 (MR1) [16]. Recently, Jeffery et al. published a comprehensive analysis of human MAIT cells in the liver, describing their pattern of distribution, association with specific diseases, and ex vivo biological properties [25].

TCR Vα7.2-Jα33-positive cells predominantly localize around the bile ducts in the portal tracts, both in normal and in diseased livers; the overall frequency of these cells increases in PSC compared to other diseases. On the contrary, in acute non-viral liver failure, the parenchymal infiltration of Vα7.2-Jα33+ cells was prominent. An analysis by flow cytometry confirmed that the majority of Vα7.2-Jα33+ cells were indeed $CD3^+CD161^+$ MAIT cells; the prevalence of MAIT cells among the total $CD3^+$ T cells either in the liver or in blood was decreased in liver diseases. Chemokine receptors that are implicated in the recruitment toward BECs, that is, CXCR6, CCR6, and integrin αEβ7, were expressed on MAIT cells in both normal and diseased livers, whereas CXCR3, being responsible for the sinusoidal infiltration through its interaction with interferon-dependent ligands, such as CXCL9/10/11, was significantly upregulated in MAIT cells from diseased livers. Finally, the effector functions of MAIT cells in the liver were analyzed ex vivo by either (1) intracellular staining of cytokines and granzymes by flow cytometry or (2) cell activation assay. MAIT cells in the liver showed high frequencies of IFN-γ (55%) and TNF-α expression (89%) and low frequencies of IL-17 production (3.5%), but IL-22 and Th2 cytokines including IL-4, IL-5, and IL-13 were barely detected; as in the literature for mouse MAIT cells, distinctive subsets of human MAIT cells can produce IFN-γ and IL-17 [26]. The selective activation of blood-derived MAIT cells, but not $V\alpha7.2\text{-}CD161^-$ cells, was observed in the co-culture with BECs and *E-coli*; an increased expression of CD107a and IFN-γ was MR1 dependent, but blocking the cytokines IL-12 and IL-18 had no effect.

These results collectively demonstrate the role of MAIT cells as the first-line dominant immune surveillance effector around BECs in the human liver. A relative increase in the number of MAIT cells in the portal tract of PSC might be associated with a breach of tissue integrity in the bile duct by microorganisms. Furthermore, the inflammatory contribution of MAIT cells could expand beyond the portal tract to the sinusoidal location through certain chemokine gradients, especially in acute lobular stress in the liver. The cross talk and relay among distinct innate immune cells, which originally occupy distinct niches in the liver, e.g., lr-NK cells and MAIT cells, add further complexity to the innate immunity-related pathogenesis in cholangiopathy (Table 3.2).

As for other unconventional T cells, the number of $CD57^+CD3^+$NKT cells was reported to be increased around the injured intralobular bile duct in the human PBC liver [27], whereas that of γδ T cells was higher in the portal infiltrates in AIH, PSC, and PBC [28].

**Table 3.2** NK cell and MAIT cell in the human liver

| | Location | Chemokine receptor | References |
|---|---|---|---|
| Normal liver | | | |
| $CD56^{bright}$ NK cell | Hepatic sinusoids | CCR5/CXCR6 | [16, 22] |
| $CD56^{dim}$ NK cell | Hepatic sinusoids | $CX_3CR1$ | [22] |
| $CD3^-CD49a^+CD56^+$ NK cell | n.d. | n.d. | [22] |
| $CD161^{bright}$ $CD3^+$ MAIT cell | Portal tract (around the bile duct) | CXCR6, CCR6 | [25] |
| Diseased liver | | | |
| CD56(-)CD16(+) NK cell (biliary atresia) | Portal tract (around the bile duct) | $CX_3CR1$ | [24] |
| Cd56(+) NK cell (PBC) | Portal tract (around the bile duct) | n.d. | [19] |
| $CD161^{Bright}$ $CD3^+$ MAIT cell (PSC) | Portal tract (around the bile duct) | CXCR6, CCR6 | [25] |
| $CD161^{Bright}$ $CD3^+$ MAIT cell (acute non-viral liver failure) | Hepatic sinusoids | CXCR3 | [25] |

### *3.4.3 ILC Cells*

Recognizing that IL-33, a well-known Th2-promoting tissue signal for the activation of ILC2 cells, was increased in the sera of patients with biliary atresia, Li et al. confirmed the role of ILC2s in the tissue repair of rotavirus-induced biliary atresia in mice [29]. In this model IL-33 expression was induced in the hilar extrahepatic bile duct (EHBD), and ILC2s were recruited and infiltrated therein. ILC2s released high levels of IL-13 that in turn promoted hyperplasia of the hilar bile duct, but not the peripheral small duct cholangiocytes; long-term exogenous administration of IL-33 promoted epithelial metaplasia and facilitated carcinogenesis in the mice with bile duct-specific active Akt (myr-Akt) and Yap (YapS127A) transgenes. As the biological effects of the IL-33/ILC2/IL-13/ circuit around BECs were specifically restricted to EHBDs and hilar bile duct branches, the relative contributions of ILCs in the different forms of cholangiopathy should be examined in the future. Moreover, even though biliary atresia is regarded as a Th1-driven disease, establishment of the epithelial repair mechanisms driven by IL-33 and ILC2 in the model mice strongly implicated multilayered involvement of ILCs in cholangiopathy.

### *3.4.4 Tissue-Resident Adaptive Memory T Cells*

Recently, special attention has been paid to long-lived $CD4^+$ and $CD8^+$ $T_{RM}$, which cohabitate with the innate and innate-like lymphocytes at mucosal barrier tissue [30]. $T_{RM}$ could act as an antigen-specific sensor, generating a non-specific but rapid and tissue-wide state of alarm, resulting in amplification of tissue-resident

nonadaptive lymphocytes, as well as recruiting circulating immune cells. Though $T_{RM}$ in the human liver has yet to be characterized, insufficient cell-autonomous innate immunity of BECs could be a trigger for the activation of both innate lymphocytes and $T_{RM}$, giving rise to the amplified and complex immunological universe of cholangiopathy. Again, it should be emphasized that the innate-to-adaptive sequence in lymphocyte activation could be revised or complemented by an adaptive-to-innate relay, even in cholangiopathy.

## 3.5 Perspectives and Conclusion

The association between inborn defects impacting PAMP/PRR signaling-related molecules and cholangiopathy has not yet reported so far; neither autosomal-recessive MyD88 nor autosomal-dominant TLR3 deficiency was reported to be related to the risk of recurrent cholangitis. Gain-of-function mutation or single nucleoside polymorphisms of quantitative trait genes (QTLs) among the genes for PAMP/PRR signaling-related molecules should be investigated to gain proof of concept of the causative role of innate immunity for cholangiopathy.

On the other hand, identifying and clinically validating immunological druggable molecules which affect the cellular innate immunity around the bile duct, such as $CX_3CR1$ and CXCL1, could be a proof of concept of the causative role of innate cellular immunity for cholangiopathy, which remains intractable with current treatment options (Table 3.3).

Single-cell transcriptomal and proteomic analysis to finely dissect inflammatory cellular heterogeneity within cholangiopathy is much anticipated in the future.

**Table 3.3** Molecules associated with innate immunity in cholangiopathy

| |
|---|
| *TLRs* |
| TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 |
| RIG-I |
| MDA-5 |
| *Inhibitory molecules for TLR signaling* |
| PPAR-γ |
| CFTR |
| IRK-M |
| CIS |
| MicroRNA |
| *Humoral factors* |
| IgA, defensins |
| IL-1β, IL-6, IL-23, IL-33, TNF-α |
| IFN-β1, IFN-γ |
| IL-8, MCP-1, MIP-3α, CXCL16, CX3CL1 |
| PDGF, CTGF, TGF-β, endothelin-1 |

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# Chapter 4
# Cellular Senescence and Biliary Disorders

**Motoko Sasaki**

**Abstract** Cellular senescence is defined as a permanent growth arrest caused by several cellular injuries such as oncogenic mutations and oxidative stress. Interestingly, senescent cells are not only results of cellular damage but also may play important roles in modulating inflammation and carcinogenesis via production of senescence-associated secretory phenotypes (SASPs) including various cytokines and chemokines. Cellular senescence may be involved in the pathophysiology of various liver diseases including cholangiopathy, a category of chronic liver diseases in which cholangiocytes are affected. Primary biliary cholangitis (PBC) is a representative cholangiopathy in which cellular senescence participates in the acceleration of inflammation and progressive ductopenia. Cellular senescence may be due to dysregulated autophagy, which may cause autoimmune process via abnormal expression of mitochondrial antigen in PBC. An involvement of cellular senescence is also reported in other cholangiopathies such as primary sclerosing cholangitis and biliary atresia. Furthermore, "oncogene-induced senescence" (OIS) may participate in multistep carcinogenesis of cholangiocarcinoma. Cellular senescence could be promising targets for prevention, early diagnosis, and therapy of various cholangiopathies in near future.

**Keywords** Cellular senescence • Oncogene-induced senescence • Senescence-associated secretory phenotypes (SASPs) • Cholangiopathy • Primary biliary cholangitis • Primary sclerosing cholangitis • Cholangiocarcinoma

## 4.1 Introduction

Cellular senescence is defined as a permanent growth arrest caused by several cellular injuries such as oncogenic mutations and oxidative stress [1, 2]. Cellular senescence is a potent antitumor mechanism [1, 2]. Recent progress revealed that cellular senescence may be involved in pathophysiology in various nonneoplastic

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inflammatory diseases [1–7]. Furthermore, "oncogene-induced senescence" or cellular senescence caused by DNA damage may occur in the process of multistep cholangiocarcinogenesis, and escape and/or bypass from senescence is important for the development of overt carcinoma [2, 8, 9]. Interestingly, senescent cells are not only results of cellular damage but also may play important roles in modulating inflammation and carcinogenesis via production of senescence-associated secretory phenotypes (SASPs) such as various cytokines and chemokines [1, 10–13].

Cholangiopathy is a category of chronic liver diseases in which cholangiocytes are damaged [6, 14, 15]. Cholangiopathy includes various diseases such as primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), cystic fibrosis, biliary atresia, polycystic disease, and cholangiocarcinoma [6, 14, 15]. We have firstly disclosed an involvement of cellular senescence and possible roles in the modulation of inflammation via SASPs and in the pathogenesis of progressive bile duct loss in PBC [4–6, 10]. Recent studies reported an involvement of cellular senescence in other cholangiopathies such as PSC and biliary atresia, shedding light again on the roles of cellular senescence in cholangiopathy [6, 7, 16, 17]. Furthermore, cellular senescence may be involved in the pathophysiology of cholangiocarcinomas [9, 18–20]. We have reported that "oncogene-induced senescence" was induced in the early stage of cholangiocarcinogenesis arising in both large and small bile ducts and that the expression of EZH2 may be a key factor for bypass/escape from cellular senescence [9, 18–20].

Herein, we review the latest studies regarding cellular senescence in the pathophysiology of cholangiopathy, putting emphasis on PBC.

## 4.2 Cellular Senescence

Cellular senescence essentially refers to an irreversible growth arrest caused by various cellular stress and injuries [1, 2]. Cellular senescence is a potent tumor suppression mechanism as well as apoptosis and is thought as a mechanism balancing cancer and aging [2, 8]. Senescent cells remain metabolically active, even though they are irreversibly arrested at the G1 phase of the cell cycle and do not respond to various external stimuli. Senescent cells are seen in aged and/or damaged tissues, which may decline tissue regeneration capacity with age [1, 2], and they may limit wound-healing responses following tissue damage [21]. Accumulating data suggest that cellular senescence may play a role in the pathophysiology of various hepatobiliary diseases and also cholangiocarcinogenesis [4–6, 19, 21–24].

### *4.2.1 Replicative Senescence and Stress-Induced Senescence*

There are two types of cellular senescence, replicative senescence and stress-induced or premature senescence. Normal cells can only divide a finite number of times before they reach a state of replicative cellular senescence [1, 2]. Telomere

shortening due to cell division is thought to be critical to induce replicative senescence. In contrast, premature or stress-induced senescence can be induced by various cellular stresses before cells reach a state of replicative senescence [1, 2]. Cellular senescence can be triggered by a number of cellular stresses including repeated cell division and strong mitogenic signals, telomere dysfunction, DNA damages, oncogenic mutations, protein aggregations, oxidative stress, and so on [1, 2]. OIS induced by oncogene mutations such as KRAS and BRAF mutations is one of the stress-induced or premature senescences.

### *4.2.2 Senescent Cell Markers*

Senescent cells are characterized by several features, such as histological changes in vitro and in vivo, shortened telomeres, increased activity of senescence-associated β-galactosidase (SA-β-gal), and increased expression of $p16^{INK4}$ and $p21^{WAF1/CIP1}$ (Fig. 4.1) [25]. SA-β-gal may be most widely used as marker for detecting senescent

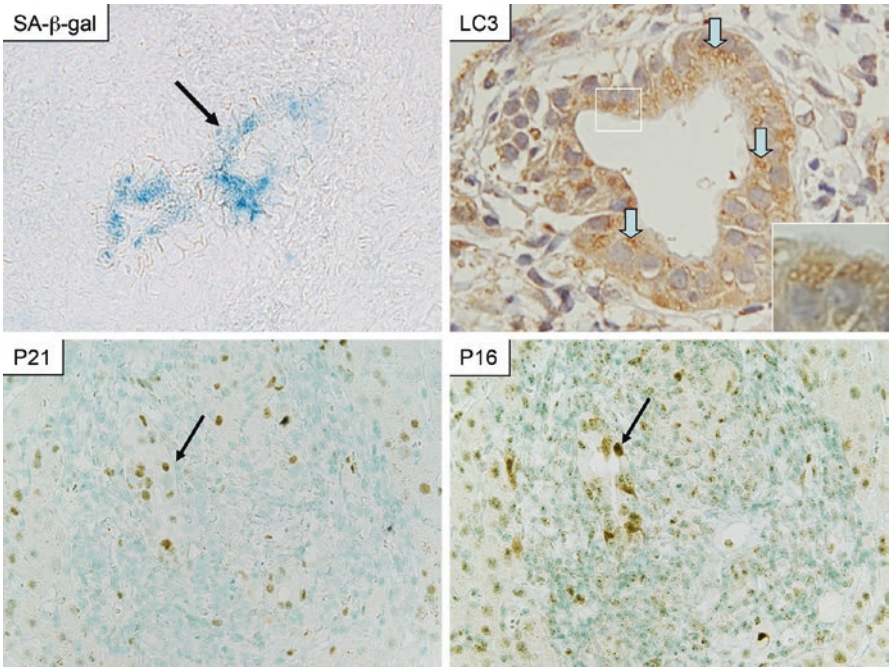


**Fig. 4.1** Cellular senescence and deregulated autophagy in the bile duct lesion in primary biliary cholangitis (PBC).
SA-β-gal activity is detected in cholangiocytes in the bile duct lesion in PBC (*top left*). Senescent markers $p21^{WAF1/CIp1}$ and $p16^{INK4a}$ were expressed in BECs in damaged small bile ducts in PBC (*bottom*). Vesicular expression of LC3 suggesting abnormal accumulation of autophagosomes was observed in damaged small bile ducts in PBC (*top right*). Immunostaining for LC3 and $p21^{WAF1/CIp1}$ and $p16^{INK4a}$. Original magnification, ×400 (inset, ×1,000)

cells in vivo and in vitro. Immunostaining for $p16^{INK4}$ and $p21^{WAF1/CIP1}$ may be also useful to detect senescent cells in tissue sections [4, 5]. Since SA-β-gal is occasionally positive for non-senescent cells, a combination of SA-β-gal with expression of $p16^{INK4}$ and $p21^{WAF1/CIP1}$ is recommended. Quantitative in situ hybridization (Q-FISH) using a telomere-specific probe is a powerful method to assess the telomere length in tissue sections [5, 26]. The strength of Q-FISH method is that it can analyze the relationship between the telomere length and histological findings in situ [5, 26].

### *4.2.3 Senescence-Associated Secretory Phenotypes (SASPs)*

Recent studies disclosed that senescent cells are not only results of cellular damage but also have a capacity to modulate the microenvironment such as inflammation, fibrosis, angiogenesis, and tumor progression by secreting biologically active molecules called SASPs (Fig. 4.2) [1, 11–13, 27, 28]. SASPs include various chemokines (CCL2/monocyte chemotactic protein-1 [MCP-1]. CXCL8/IL-8, CX3CL1/fractalkine, and so on), cytokines (IL-1, IL-6, and so on), growth factors, and profibrogenic factors (Table 4.1) [1, 11–13, 27, 28]. Cellular senescence is a double-edged sword, and SASP can have both positive and negative effects, depending on context. The SASP can cause local and potentially systemic inflammation, disrupt tissue architecture, and stimulate growth of nearby malignant cells in some situation (Fig. 4.2) [1, 2, 11–13]. The SASP may promote immune clearance of the damaged cells and alert nearby cells to potential danger. Furthermore, matrix metalloproteinases (MMPs) as SASPs can limit fibrosis following liver injury or during skin wound healing. IL-6 and IL-8 as SASPs may play a role in defense against cancer by reinforcing the senescence growth arrest [1]. Senescent cells in cholangiopathies are also thought to play a role in modulating microenvironment around the bile ducts (Fig. 4.2).

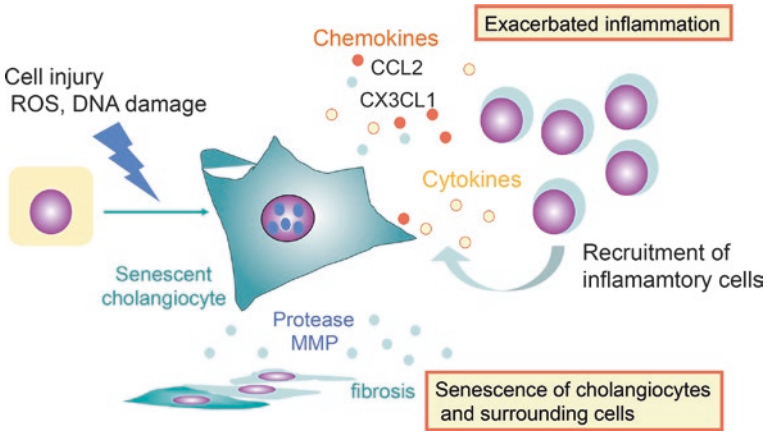


**Fig. 4.2** Cellular responses/cell death: autophagy, necrosis, apoptosis, and cellular senescence to cellular stress in cholangiocytes

**Table 4.1** Senescence-associated secretory phenotype (SASP)

| Type | Factors |
|---|---|
| Interleukin | IL-6, IL-1α, IL-1β, IL-24 |
| TNF ligand | TNFSF15 |
| Chemokine | IL-8, GROα, β, γ, CXCL20, CCL3, MCP-1 |
| Chemokine receptors | CXCR1, CXCR2 |
| Growth factors | Epiregulin, neuregulin, amphiregulin |
| TGF-β family | BMP2, activin A |
| IGFBPs | IGFBP2, IGFBP5, IGFBP7 (IGFBP-rP1) |
| Matrix metalloproteinase | MMP3, MMP14 |
| Protease inhibitors | PAI-1, PAI-2 |
| Extracellular protease | uPA, tPA |
| Collagen | Col10A1 |

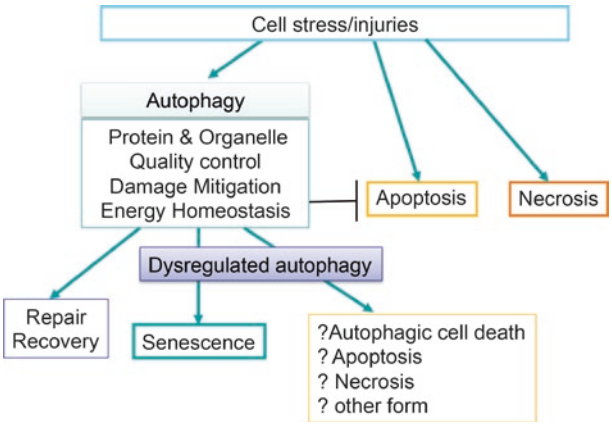


**Fig. 4.3** Modulation of microenvironment by senescent cholangiocytes via senescence-associated secretory phenotypes (SASPs) in PBC
Senescent cholangiocytes may function in modulation of the inflammatory microenvironment by recruiting monocytes and possibly other inflammatory cells by secreting chemokines and cytokines as SASPs. Senescent cholangiocytes may also participate in the induction of senescence in surrounding cells and progression of fibrosis via SASPs

## 4.3 Cellular Senescence, Autophagy, and Apoptosis as Stress Response

Cells respond to stress with repair, adaptation, and autophagy or go into cellular senescence, apoptosis, or necrosis (Fig. 4.3) [29, 30]. An appropriate cellular stress response is critical for maintaining tissue integrity and the function and prevention of diseases [29, 30]. Apoptosis, autophagy, and cellular senescence are also essential mechanisms for tumor suppression removing potentially dangerous cells with

genetic alteration such as mutations. Autophagy is a lysosomal pathway that degrades and recycles intracellular organelles such as mitochondria and proteins to maintain energy homeostasis during times of nutrient deprivation and to remove damaged cell components caused by various stresses [31, 32]. Accumulating evidences suggest physiological roles of autophagy in health and pathological participation of impaired autophagy in various diseases including cancer and neurodegenerative disorders [29, 31]. Autophagy is also known to play important roles both in innate and acquired immune systems and also in autoimmunity [33, 34]. It is reported that autophagy precedes the process of senescence and facilitates cellular senescence [35, 36]. Cellular senescence may be caused as a result of dysregulated autophagy.

## 4.4 Cellular Senescence in Cholangiopathy

Recent progress in the field of hepatology has disclosed that cellular senescence is involved in the pathophysiology of cholangiopathies and hepatocarcinogenesis [4–7, 19, 21–24]. Table 4.2 summarizes involvement of cellular senescence in cholangiopathies. Cellular senescence is thought to play a role in pathophysiology of cholangiopathy mainly from three aspects: (1) impaired regeneration of bile duct due to cell cycle arrest, (2) modulation of microenvironment via SASPs, and (3) cellular senescence as tumor-suppressing system (oncogene-induced senescence).

**Table 4.2** Review of literature on an involvement of cellular senescence in cholangiopathy

| | Sites of cellular senescence: senescent marker used | Significance of cellular senescence | Refs. |
|---|---|---|---|
| PBC | Intrahepatic small bile duct, bile ductules: SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/Cip1}$, telomere shortening | Bile duct loss due to impaired regeneration Exacerbation of inflammation and accelerated induction of senescence by SASPs | [6, 7] |
| PSC | Bile duct: SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/Cip1}$ | Exacerbation of inflammation and accelerated induction of senescence by SASPs | [16] |
| Chronic rejection | Bile duct: $p21^{WAF1/Cip1}$ | Bile duct loss due to impaired regeneration | [6, 37] |
| Biliary atresia | Bile duct, hepatocyte: SA-β-gal, $p16^{INK4a}$, $p21^{WAF1/Cip1}$, telomere shortening | Bile duct loss due to impaired regeneration | [17, 38] |
| Cholangiocarcinoma; precursor lesions | Biliary intraepithelial neoplasia (BilIN): $p16^{INK4a}$ Pancreatobiliary maljunction; hyperplastic lesions: SA-β-gal, $p16^{INK4a}$ | Oncogene-induced senescence (OIS) | [4, 39] |

*SA-β-gal* senescence-associated β-galactosidase, *SASPs* senescence-associated secretory phenotypes

Representatively, cellular senescence is involved in bile duct lesions in PBC and ductular reaction (DR) in various chronic advanced liver diseases, as described below [4–7, 10, 40, 41].

### *4.4.1 PBC*

PBC is a representative inflammatory cholangiopathy characterized by a high prevalence of serum anti-mitochondrial antibodies (AMAs) against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) and several other mitochondrial enzymes [6, 14]. PBC is histologically characterized by cholangitis in the small bile ducts (chronic nonsuppurative destructive cholangitis, CNSDC), eventually followed by extensive loss of small bile ducts and biliary cirrhosis [42, 43]. Although PBC is regarded as an autoimmune-mediated liver disease, exact autoimmune mechanisms and the significance of AMAs have not been clarified, so far. These days, early diagnosis of PBC has dramatically improved with measurement of markers of cholestasis and serum AMAs. Prognosis of patients with PBC is also improved with the introduction of ursodeoxycholic acid (UDCA) treatment and the introduction of liver transplantation [15]. Based on recent alterations of this disease entity and requests by patients' group, the proposal of changing the name "primary biliary cirrhosis" to "primary biliary cholangitis" has been recently approved worldwide [44, 45].

#### 4.4.1.1 Biliary Epithelial Senescence in Damaged Small Bile Duct in PBC

We have reported the cellular senescence of cholangiocytes with shortened telomeres, the expression of SA-β-gal, and the augmented expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in damaged small bile ducts involved in CNSDC in PBC (Fig. 4.1) [4, 5]. This suggests that cellular senescence may be involved in the pathogenesis of PBC from two aspects: progressive bile duct loss and modulation of microenvironment via secretion of SASPs in PBC. Oxidative stress due to inflammation may play a role for the induction of cellular senescence [4, 22, 46, 47].

#### 4.4.1.2 Cellular Senescence and Bile Duct Loss in PBC

The exact mechanism how cellular senescence of cholangiocytes causes bile duct loss in PBC is not clear. Cellular senescence is supposed to impair tissue integrity and cause persistent inflammation [48]. After cellular senescence occurs in injured cholangiocytes, these senescent cells are thought to remain in situ and not to be replaced by normal cells, although non-senescent cholangiocytes proliferate in response to injury [4, 5, 49]. Senescent cholangiocytes may be prone to further injuries caused by accentuating inflammation due to SASP, which is likely to be

followed by bile duct loss in PBC. The fate of senescent cholangiocytes remains to be clarified; whether senescent cholangiocytes are removed by necrosis, apoptosis, anoikis, or other types of regulated cell death. Cellular senescence is also seen in bile ductular cells in a ductular reaction (DR), which is thought to harbor hepatic stem/progenitor cells in PBC [4, 5, 40]. So, the impaired proliferation of hepatic stem/progenitor cells may fail to replace the damaged cholangiocytes in small bile ducts and subsequently caused bile duct loss.

#### 4.4.1.3 Modulation of Inflammation by SASPs in PBC

Furthermore, senescent cholangiocytes may modulate inflammatory microenvironment around affected small bile ducts by recruiting monocytes and possibly other types of inflammatory cells via SASPs, such as CCL2 and CX3CL1 in PBC (Fig. 4.2) [6, 7, 10, 50]. The expression of CCL2 and CX3CL1 was co-localized with the expression of senescent markers in damaged bile ducts in PBC [10]. In culture study, senescent cholangiocytes induced by cellular stresses expressed a significantly higher level of chemokines. Furthermore, senescent cholangiocytes significantly accelerated the migration of RAW264.7 cells (macrophage/monocyte), and neutralizing antibodies against CCL2 and CX3CL1 blocked in part the migration induced by senescent cholangiocytes [10]. It has been reported that cholangiocytes express a number of profibrogenic proinflammatory and chemotactic factors (e.g., IL-1, IL-6, CXCL8/IL-8, and CCL2/MCP-1) at the bile duct lesion in PBC [51–54]. Such cytokines and chemokines seem to belong to SASPs [1, 11–13, 27, 28]. These factors can attract and activate inflammatory cells and also stellate cell lineage in humans with biliary disorders and in animal models of biliary fibrosis.

#### 4.4.1.4 Cellular Senescence in Ductular Reaction

Ductular cells in ductular reaction in the advanced stage of various chronic liver diseases, including PBC and nonalcoholic steatohepatitis (NASH), frequently express senescence-associated $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ [4, 5, 40]. Cellular senescence of ductular cells expressing $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ is most prominent in PBC at the advanced stages [40]. Ductular cells also express cyclin D in parallel with the expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$, suggesting G1-arrest and undergoing cellular senescence [4, 5, 40]. Severely impaired regeneration due to cellular senescence in ductular cells may be responsible for bile duct loss in PBC. Furthermore, it is likely that senescent bile ductular cells in ductular reactions may play a role in the progression of fibrosis in chronic liver diseases [5, 40]. For example, bile ductular cells in ductular reactions express CCL2 as SASP, which may be responsible for chemoattraction of activated hepatic stellate cells (HSCs) and inflammatory cells and subsequent fibrosis in PBC and NASH [41, 55]. In culture study, senescent mouse cholangiocytes significantly facilitated the migration of HSCs by secretion of CCL2 [41].

#### 4.4.1.5 Cellular Senescence and Dysregulated Autophagy

Accumulating evidences suggest that dysregulated autophagy may be a central player in the pathogenesis of PBC (Fig. 4.4) [6, 36, 56, 57]. Dysregulated autophagy may precede cellular senescence in bile duct lesions in PBC [36, 56]. Furthermore, dysregulated autophagy may be related to autoimmune process against mitochondrial antigens in PBC [57]. We disclosed the accumulation of autophagy marker LC3-positive vesicles in the damaged small bile ducts in the bile duct lesion (CNSDC) in PBC (Fig. 4.1) [36]. LC3 was characteristically expressed in cytoplasmic vesicles in bile duct lesions in PBC, which suggests "abnormal accumulation of autophagosome" due to impaired autophagy [36]. Furthermore, the aggregation of dysregulated autophagy marker p62 is specifically increased together with the accumulation of LC3-positive vesicles in damage bile ducts in PBC [56]. The accumulation of autophagic vacuoles at various stages in autophagic process was ultrastructurally confirmed in cholangiocytes in damaged bile ducts in PBC [56].

Dysregulated autophagy seems to be related to the induction of senescence, and the inhibition of autophagy delays the senescence phenotype [35, 36]. Autophagic marker LC3 was co-expressed with senescent markers $p21^{WAF1/Cip1}$ and $p16^{INK4a}$ in damaged bile ducts in PBC [36]. In vitro study supported the involvement of autophagy in cellular process of stress-induced cellular senescence in cholangiocytes [36]. Dysregulated autophagy may further contribute to increase intracellular ROS due to impaired removal of damaged mitochondria in cholangiocytes. Although exact mechanisms of deregulated autophagy remain unclear, an involvement of endoplasmic reticulum (ER) stress induced by GCDC in cholangiocytes is suggested [58].

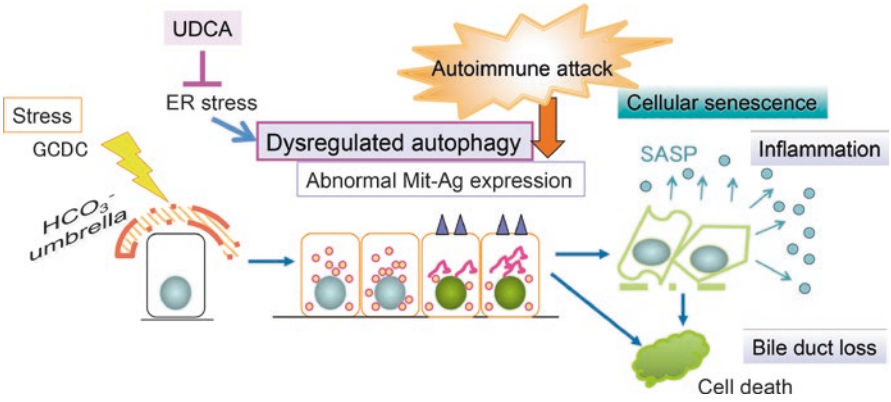


**Fig. 4.4** Dysregulated autophagy may be a central player in the pathogenesis of PBC in two aspects: autoimmune process against mitochondrial antigens and the induction of cellular senescence in cholangiocytes. *ER* endoplasmic reticulum, *GCDC* glycochenodeoxycholic acid, *SASP* senescence-associated secretory phenotypes, *UDCA* ursodeoxycholic acid

#### 4.4.1.6 Dysregulated Autophagy and Autoimmunity Toward Mitochondrial Antigens in PBC

Autoimmune mechanisms of PBC, especially a significance of AMAs, have not been full clarified, so far. Since mitochondria are a major target of autophagy, we hypothesized that dysregulated autophagy of mitochondria may be involved in autoimmune pathogenesis in PBC [59]. We found that granular expression of PDC-E2 was co-localized with LC3 in damaged small bile ducts in PBC [59]. This type of PDC-E2 expression was not seen is other control diseased liver, such as chronic viral hepatitis [59]. These findings suggest that the PBC-specific abnormal accumulation of mitochondrial antigen due to dysregulated autophagy may be related to autoimmune reaction toward mitochondrial antigens in PBC (Fig. 4.4) [59].

### *4.4.2 PSC*

PSC is an inflammatory and fibrosing cholangiopathy affecting intrahepatic and extrahepatic large bile ducts [6, 14, 15]. PSC has a prominent association with inflammatory bowel disease in 70 % of patients. PSC sometimes progresses to biliary cirrhosis and is associated with cholangiocarcinoma. Recently, an involvement of cellular senescence in the pathophysiology of PSC has been reported [16]. That is, expression of senescent markers, $p16^{INK4a}$, $p21^{WAF1/Cip1}$, and SA-β-Gal, was observed in cholangiocytes in large bile ducts in PSC. However, telomere shortening assessed by Q-FISH methods was not proven in PSC, suggesting complex mechanisms of cellular senescence [16]. In PSC, cellular senescence was reportedly induced via NRAS activation and LPS and secretion of SASPs [16]. Interestingly, PSC mouse model MDR2 knockout mice showed cellular senescence in bile ducts [60]. Furthermore, recent study reported association of gut microbiota in the induction of cellular senescence in MDR2-/- mice [60].

### *4.4.3 Biliary Atresia*

Biliary atresia is a pediatric cholangiopathy in which progressive fibrous obstruction of intrahepatic and extrahepatic bile ducts occurs [14]. Biliary atresia is the most common cause of pediatric liver transplantation. A combination of viral, toxic, genetic, and immunological etiologies has been implied, but the pathophysiology of biliary atresia remains unknown. In biliary atresia, expression of senescent markers, $p16^{INK4a}$, $p21^{WAF1/Cip1}$, and SA-β-Gal, was observed in bile ductules and hepatocytes [17]. Telomere shortening was also detected in hepatocytes by Q-FISH in biliary atresia [17]. Furthermore, increased expression of IGFBP-rP1 as a SASP was observed, suggesting participation of cellular senescence in the pathophysiology of biliary atresia [38].

### 4.4.4 Chronic Rejection

"Vanishing bile duct syndrome" is a category of liver disease in which progressive bile duct loss and chronic cholestasis occur. "Vanishing bile duct syndrome" includes PBC, PSC, and chronic rejection after liver transplantation, and some common mechanism causing "vanishing bile duct syndrome" has been speculated. Cholangiocytes in early stage of chronic rejection show histological features of cellular senescence with increased expression of senescence-related $p21^{WAF1/CIP}$ [4, 37, 61]. Senescent cells are known to contribute to an impaired tissue integrity due to impaired cell proliferation after cellular damage and persistent inflammation [48]. Upregulated expression of TGFβ by some immunosuppressive drugs may enhance cellular senescence [48]. That is, patients with failed allografts and cyclosporine had more bile duct loss than patients with tacrolimus [62]. The increased expression of senescence-related $p21^{WAF1/Cip1}$ protein decreases with successful recovery in cholangiocytes during early chronic rejection [37].

## 4.5 Cellular Senescence in Cholangiocarcinogenesis

Recent progress revealed that cellular senescence might be involved in the pathophysiology of cholangiocarcinomas [9, 18–20]. "Oncogene-induced" and/or stress-induced senescence may occur in the process of multistep cholangiocarcinogenesis in perihilar cholangiocarcinomas via precursor lesion, biliary intraepithelial neoplasia (BilIN), in hepatolithiasis [9]. Overexpression of a polycomb group protein EZH2 is thought to play a role in escape and/or bypass from senescence [9, 18, 39]. Similarly, cellular senescence and bypass/escape from senescence may be involved in the carcinogenesis of cholangiolocellular carcinoma, a subtype of peripheral-type intrahepatic cholangiocarcinomas [18]. Furthermore, senescent cells in precursor lesions and cells around cholangiocarcinomas may play important roles in tumor development and progression via production of SASPs. Cellular senescence may be a new target for prevention, early diagnosis, and therapy of cholangiocarcinoma [9, 18–20].

### 4.5.1 Cholangiocarcinoma, Perihilar Type, and Precursor Lesions

BilIN is thought to be a flat-type precursor or early neoplastic biliary lesion [63, 64]. BilIN is frequently found in intrahepatic large bile ducts and perihilar bile ducts and peribiliary glands in hepatolithiasis [63–67]. The cellular senescence may reflect OIS in hepatolithiasis, since KRAS mutations are detected in one-third of BilIN lesions as well as background large bile ducts and peribiliary glands in

hepatolithiasis [68]. That is, the expression of senescence-associated $p16^{INK4a}$ occurs in the early stage and then, overexpression of EZH2 plays a role in the bypass/escape from senescence followed by the development of overt carcinoma [9, 39]. $p16^{INK4a}$ promoter hypermethylation may be related to aberrant expression of EZH2, and this finding was supported by in vitro study [9].

### *4.5.2 Peripheral Intrahepatic Cholangiocarcinomas (ICCs) and Precursor Lesions*

The carcinogenesis pathways and precursor lesions regarding the peripheral type of ICCs remain unclear, so far. We found that the expression of senescence-associated $p16^{INK4a}$ and EZH2 showed interesting pattern in cholangiolocellular carcinomas, ductular reaction, and bile duct adenoma, which may reflect the involvement of cellular senescence in the carcinogenesis of cholangiolocellular carcinoma [18, 19]. An extensive expression of senescence-associated $p16^{INK4a}$ in bile duct adenomas and ductular reactions seems to represent premalignant features with genetic alterations such as KRAS mutation (OIS) [8]. Furthermore, EZH2 may play a role in bypass/escape of cellular senescence during progression of cholangiolocellular carcinoma, similar to CCAs arising in the large bile ducts [9, 39]. Further studies are needed to confirm this hypothesis.

## 4.6 Summary

Accumulating evidences suggest that cellular senescence may play a role in pathophysiology of various liver diseases including cholangiopathies. Senescent cells are not only results of cellular damage but also may participate in modulating inflammation and carcinogenesis via production of SASPs including various cytokines and chemokines. Cellular senescence is thought to be involved in augmented inflammation and progressive ductopenia in PBC. Dysregulated autophagy may be a central player in the pathogenesis of PBC by inducing cellular senescence and autoimmune process via abnormal expression of mitochondrial antigen. Furthermore, “oncogene-induced senescence” may participate in multistep carcinogenesis of cholangiocarcinoma. Cellular senescence can be a new target for prevention, early diagnosis, and therapy of various cholangiopathies in the near future.

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# Chapter 5
# Vascular Supply of the Bile Duct and Ischemic Cholangiopathy

**Yasuni Nakanuma and Naoko Miyata**

**Abstract** The biliary tree is exclusively supplied by the hepatic artery, so hepatic arterial insufficiency and also the damages to the hepatic arteries and their branches including peribiliary vascular plexus (PVP) give rise to various forms of ischemic biliary damages (ischemic cholangiopathies). Ischemic cholangiopathies are composed of several clinicopathological categories such as bile duct necrosis, bile leak and biloma, biliary strictures, and biliary casts. Ischemic cholangiopathies related to liver transplantation including nonanastomotic ischemic-type biliary stricture and biliary cast syndrome and also those associated with transarterial chemoembolization including bile duct necrosis and biloma are well known, though ischemic cholangiopathies also occur spontaneously and in other medical treatments. Hypoxic injuries to hepatic arterial branches including PVP and biliary epithelial cells (BECs) seem to be essential. In addition, ABH blood type and HLA antigens expressing on endothelial cells in the PVP and BECs in the large bile ducts could be targets by an immunological attack followed by the development of ischemic cholangiopathy. Further studies on the unique anatomy of the bile ducts supplied by PVP, both expressing graft-specific antigens, are essential for evaluation of and therapeutical approach to ischemic cholangiopathies.

**Keywords** Peribiliary vascular plexus • Bile duct • Biloma • Bile duct necrosis Sclerosing cholangitis

## A List of Abbreviations

| | |
|---|---|
| AS | Anastomotic biliary stricture |
| BCS | Biliary cast syndrome |
| BDN | Bile duct necrosis |
| BECs | Biliary epithelial cells |
| DSA | Donor-specific antibodies |

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| | |
|---|---|
| HR | Hypoxia/reperfusion |
| IA | Isoagglutinin |
| ITBL | Ischemic-type biliary stricture |
| LPS | Lipopolysaccharides |
| LT | Liver transplantation |
| NAS | Nonanastomotic biliary strictures |
| PAMPs | Pathogen-associated molecular patterns |
| PCP | Peribiliary capillary plexus |
| PRR | Pathogen recognition receptors |
| PVP | Peribiliary vascular plexus |
| ROS | Reactive oxygen species |
| TACE | Transarterial chemoembolization |
| TLRs | Toll-like receptors |

## 5.1 Introduction

The biliary tree lined by a single layer of biliary epithelial cells (BECs) or cholangiocytes is an excretory conduit for bile secreted from hepatocytes and BECs [1, 2]. Different from the liver parenchyma supplied dually by portal veins and hepatic arteries, the biliary tree is exclusively supplied by the hepatic artery. Therefore, it seems likely that hepatic arterial insufficiency and also the damages to the hepatic arteries and their branches including peribiliary vascular plexus (PVP) give rise to various forms of ischemic biliary damages (ischemic cholangiopathies) [1, 3].

Recently, liver transplantation (LT) and interventional therapies such as transarterial chemoembolization (TACE) become popular, and various forms of biliary complications are frequently encountered clinically, and some of them are associated with significantly higher morbidity and mortality [4–6]. A majority of these biliary complications may be causally related to biliary ischemia [4]. Particularly, nonanastomotic ischemic-type biliary lesions (ITBLs) belong to one of the most frequent and troublesome complications of LT [4, 7, 8]. Ischemic biliary injuries such as biliary strictures and cholangitis also occur spontaneously [4].

In this review, the pathologies and pathogenesis of cholangiopathies related to the disturbance of blood supply to the biliary tree are discussed with respect to the unique anatomy of blood supply to the biliary tree.

## 5.2 Blood Supply to the Biliary Tree

### *5.2.1 Anatomy of the Biliary Tree*

The biliary tract is generally divided into the distal, perihilar, and intrahepatic bile duct and gallbladder. The intrahepatic bile duct, proximal to the right or left hepatic bile duct, is composed of the intrahepatic large and small bile ducts. The former correspond to the first to third branches of both hepatic ducts, while the latter which are recognizable under a microscope are composed of septal and interlobular bile

ducts. Interestingly, the intrahepatic large bile duct and perihilar and distal bile ducts are accompanied by peribiliary glands [2]. The biliary tract is lined by a single layer of cuboidal to high columnar simple epithelium (BECs).

### 5.2.2 *Vasculatures Supplying the Biliary Tree*

The biliary tract ranging from the extrahepatic bile duct to the intrahepatic small bile ducts and also the peribiliary glands are exclusively supplied by hepatic arterial blood flow [1, 3]. Small arterial branches deriving from hepatic arteries ramify, anastomose, and surround densely the bile ducts like chicken wire mesh (peribiliary (connecting) arterioles) within the ductal wall, and these arterioles taper to form the peribiliary capillary plexus (PCP) beneath the biliary lining epithelial layer. The irrigated blood then drain into the venous branches around and within the ductal wall and then drain into the portal vein branches or directly hepatic sinusoids. The vascular structures composed of peribiliary arteries, PCP, and draining venules are called PVP [3]. Ultrastructurally, a considerable number of these capillaries are composed of fenestrated endothelium with a diaphragm and with extreme cytoplasmic attenuations that are dense at the sides facing the bile duct for efficient transport of substances to and from BECs [9].

Histologically, the PVP has three well-developed layers in large intrahepatic/perihilar and distal bile ducts (Figs. 5.1 and 5.2a) [3]: inner layer corresponding to PCP,

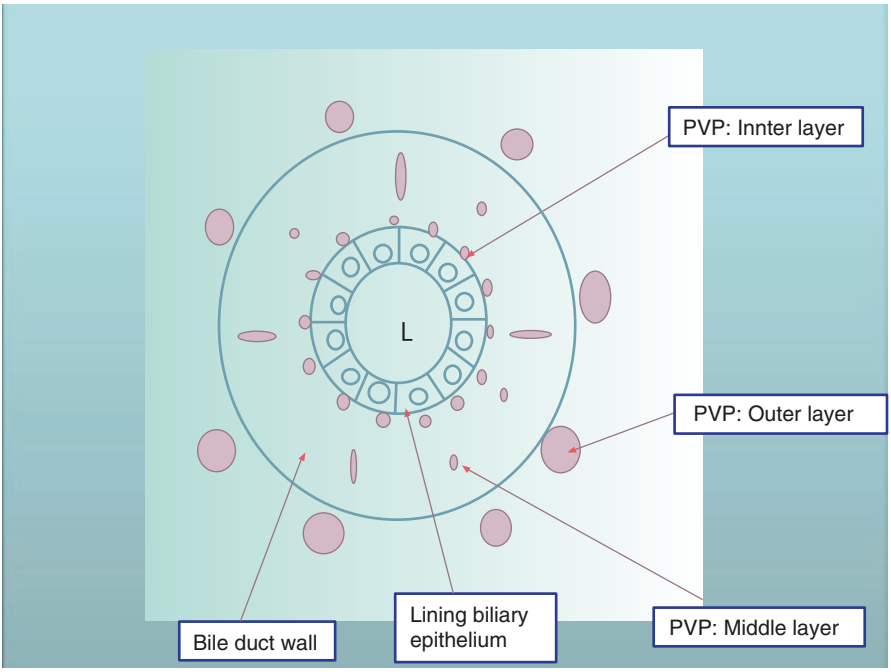


**Fig. 5.1** Schema of peribiliary vascular plexus (PVP) of the bile duct. There is a *dot*-like inner layer facing the lumen of the bile duct (L) and vasculatures outside the duct wall (outer layer). Within the duct wall, there are also small vessels (middle layer). *L* bile duct lumen

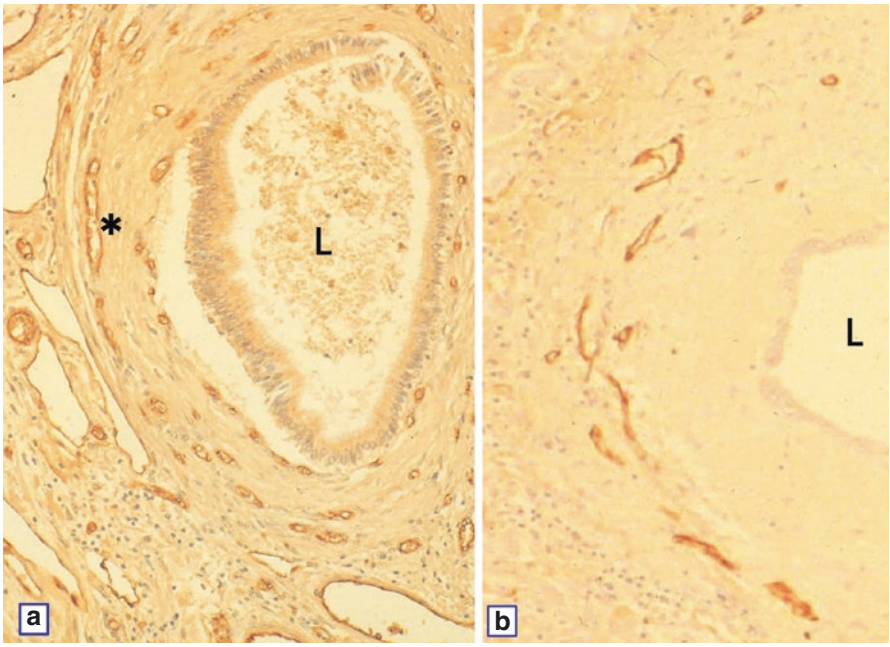


**Fig. 5.2** Peribiliary vascular plexus (PVP) of the bile duct and its alteration. (**a**) There is a *dot*-like inner layer facing the lumen of the bile duct (L) and vasculature (*arrows*) outside the duct wall (outer layer). In the duct wall (*), there are also small vessels (middle layer). *L* bile duct lumen. Immunostaining of factor IIIV., x100 (original magnification). (**b**) Bile duct sclerosis due to trans-arterial chemoembolization. Diminution or loss of inner layer is evident in the septal bile duct. *L* bile duct lumen. Immunostaining of factor IIIV., x130 (original magnification)

middle and outer layers corresponding to the afferent arterioles and small arteries, and the efferent draining venules and small veins within and outside the duct wall. This three-layer organization is evident along the distal, perihilar, and intrahepatic large bile ducts and septal bile ducts but becomes less evident around the interlobular bile duct.

## 5.3 Ischemic Biliary Epithelial Injuries and Bile Duct Pathologies

Under various ischemic conditions to the biliary tree, the bile ducts show various biliary epithelial injuries and bile duct pathologies (ischemic cholangiopathies) [10].

### *5.3.1 Mechanisms of Bile Duct Ischemia*

The following diffuse or localized ischemic conditions of the hepatic vasculatures or hepatic circulation are known to be responsible for ischemic cell injuries of BECs resulting in benign stricture and other pathologic lesions.

#### 5.3.1.1 Shock or Hypoperfusion of the Hepatic Artery

Shock and hepatic arterial insufficiency or hypoperfusion cause ischemia to the bile duct.

#### 5.3.1.2 Vascular Injuries of Hepatic Arteries and PVP

Local or segmental injuries to the hepatic arterial branches or PVP itself may lead to the focal, multifocal, segmental, or discontinuous ischemia of bile ducts.

##### Thrombosis, Embolization, or Other Mechanical Injuries

Hepatic arterial flow may be compromised by thrombosis or embolization, stenosis, or kinking of the hepatic artery followed by ischemic bile duct damage such as biliary strictures and loss.

##### Arteritis or Arteriopathy or Vasculitis

**Rejection-related arteriopathy (foam cell arteriopathy)**: Rejection-related arteriopathy consists of the presence of foamy macrophages, which are usually subintimal but may be located in any layer of the arterial wall. These cells cause variable degrees of luminal occlusion of affected arteries, which may contribute to ischemic bile duct damage and loss and may often be associated with cholestasis and bile duct damage.

**Other vascular lesions**: Necrotizing arteritis (polyarteritis nodosa) of hepatic arterial branches is a rare cause of ischemic bile duct injury. Dissecting aneurysm of the hepatic artery or its branches associated with luminal obliteration is also responsible for the occurrence of bile duct ischemia.

##### Endothelial Injuries of PVP

**Antibodies mediated attacks to PVP**: Circulating preformed donor-specific antibodies (DSA) and de novo DASs have been reported to immunologically challenge vascular endothelial cells of PVP (Fig. 5.3) [5, 11]. Blood type and HLA antigens expressed on endothelial cells in the PVP could be targeted by DSA [1, 12].

**Microangiopathic injuries to PVP**: Many cytotoxic insults such as hypoxic injuries; reactive oxygen species (ROS); bacterial toxin, particularly endotoxin; preservation-induced injuries; and cold and warm ischemic insults induce direct injuries or destruction of the endothelial cells of PVP, particularly inner and middle layer.

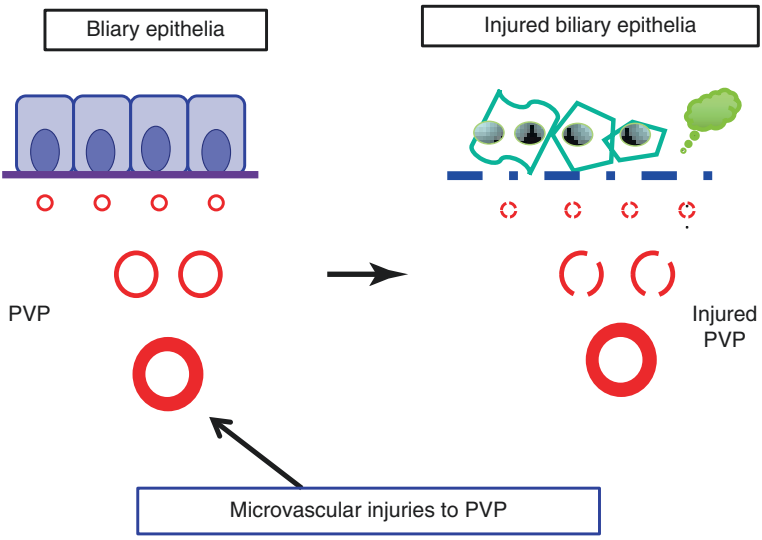


**Fig. 5.3** Schematic presentation of microvascular injuries to PVP (peribiliary vascular plexus) and resultant biliary epithelial injuries; cytotoxic injuries such as cytotoxic antibodies, endotoxin, and reactive oxygen species to the endothelium of PVP lead to disruption of PVP, particularly inner and middle layers, and then lining biliary epithelial injuries occur, followed by ischemic cholangiopathy

## *5.3.2 Ischemic Injuries of BECs*

As a consequence of bile duct ischemia, the following biliary epithelial injuries develop.

### 5.3.2.1 Hypoxia and Hypoxia/Reperfusion (H/R)

As a consequence of hypoxia, BECs lose their intercellular connections and detach from the basement membrane, resulting in sloughing of the epithelial lining and denudation of the bile duct luminal surface [13]. However, BECs are more susceptible to reperfusion injuries than to hypoxia, supporting the hypothesis that reperfusion of anoxic BECs increased cell killing of BECs and reperfusion injury during LT leads to bile duct injury after LT [6, 13]. These H/R injuries may induce apoptosis, necrosis, and cellular senescence of BECs.

### 5.3.2.2 Toxic Bile Acids

$HCO_3^-$ secretion from BECs normally maintains an alkaline pH on their luminal surface preventing the uncontrolled permeation and damage from hydrophobic bile acids which are cell injurious [13, 14]. The BEC "protector" $HCO_3^-$ secretion may

be disturbed after LT, as H/R results in altered expression of the anion exchanger 2 and of the cystic fibrosis transmembrane conductance regulator protein, which regulates the biliary secretion of $HCO_3^-$ [13, 14]. Under ischemic conditions, biliary $HCO_3^-$ formation may be impaired, and such impairment enhances BEC vulnerability toward the attack of hydrophobic bile acids (see Chap. 2) [12].

#### 5.3.2.3 Deregulated Biliary Innate Immunity

BECs are continuously exposed physiologically and pathologically to bile in the duct lumen which contains a number of endogenous and exogenous constituents, including pathogen-associated molecular patterns (PAMPs) and xenobiotics, which are generally proinflammatory, chemotactic, and cell injurious [15]. For protection of BECS, bile ducts are equipped by several cell defense systems including the unique innate immune responses (biliary innate immunity). Particularly, BECs possess pathogen recognition receptors (PRR), particularly Toll-like receptors (TLRs), which recognize PAMPs and play a pivotal role in the innate immune response. In deregulated conditions, innate immunity may be activated and secrete proinflammatory cytokines and chemokines and other bioactive materials resulting in the development of the inflammatory and fibrotic periductal cytokine milieu and the induction of biliary apoptosis in ischemic cholangiopathy (see Chaps. 3 and 4). Experimental, reperfusion-induced tissue injury is mediated by an innate immune-dominated inflammatory response, at least in part, by activation of TLR4- and probably also of TLR2-expressing cells [16].

#### 5.3.2.4 Oxidative Stress and Antioxidant

Ischemic state of the biliary tree is associated with oxidative stress. The rate of ROS formation by BECs was fivefold greater than in hepatocytes during reoxygenation. In addition, basal levels of glutathione are lower in BECs than in hepatocytes, so BECs are more susceptible to reoxygenation injury than to anoxia, supporting the hypothesis that reoxygenation injury during liver preservation leads to bile duct injury during LT. Endogenous cytoprotective molecules such as antioxidants protect BEC. However, the reduction in the cellular levels of antioxidants such as glutathione (GSH) under ischemic conditions results in increased degradation of Bcl-2 protein and an increase in apoptosis of BECs and cholangiopathies [17].

#### 5.3.2.5 Others

Prolonged cold ischemia time during LT is associated with a downregulation of membrane-associated MUC-1, MUC-3A, and MUC-5B expression [16]. Mucins are expressed on the apical membrane of the biliary epithelial cells and lubricate and protect these cells from diverse injuries, including injury by cytotoxic bile salts. Decreased expression of MUC1 and MUC3A after LT may favor the development of ITBLs.

### *5.3.3 Pathologies of Bile Ducts in Ischemia*

Biliary ischemia is involved in the development of the following bile duct lesions which are frequent in LT and TACE. However, these lesions also occur in nonischemic biliary tract.

#### 5.3.3.1 Bile Duct Necrosis (BDN)

BDN exhibits loss of viable cells in the mural stroma of the bile duct wall [8]. Although it is commonly accompanied by mucosal loss leading to denuded subepithelial stroma, the loss of epithelial lining alone is not interpreted as BDN. Typically, BDN is more pronounced at the inner mural layer, and the avital areas showed scattered cells with karyolysis or no cellular remnant at all. The degree of BDN is variable, and BDN is frequently associated with imbibition of bile and bile pigments in the necrotic area (Fig. 5.4). The necrosis of the bile duct wall is most likely caused by occlusion of small arteries and also local microenvironmental disruption of PVP associated with destruction of inner PCP.

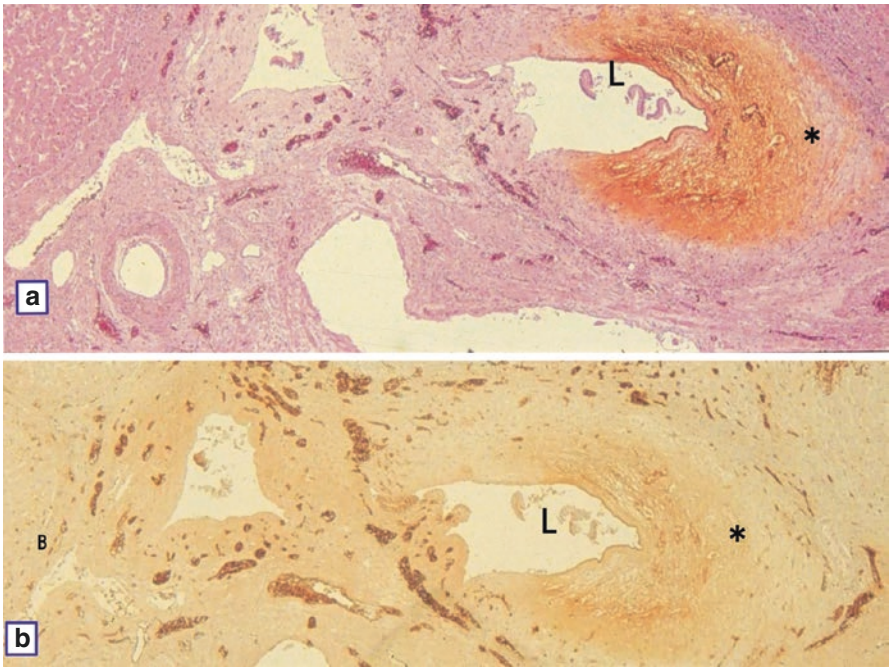


**Fig. 5.4** Bile duct necrosis due to transarterial chemoembolization. (**a**) More than a half of septal bile duct shows necrosis and bile imbibition. H&E., x70 (original magnification). (**b**) Serial section of **a**. In the necrotic area, inner and middle layers of peribiliary vascular plexus are lost. Immunostaining of factor IIIV., x70 (original magnification)

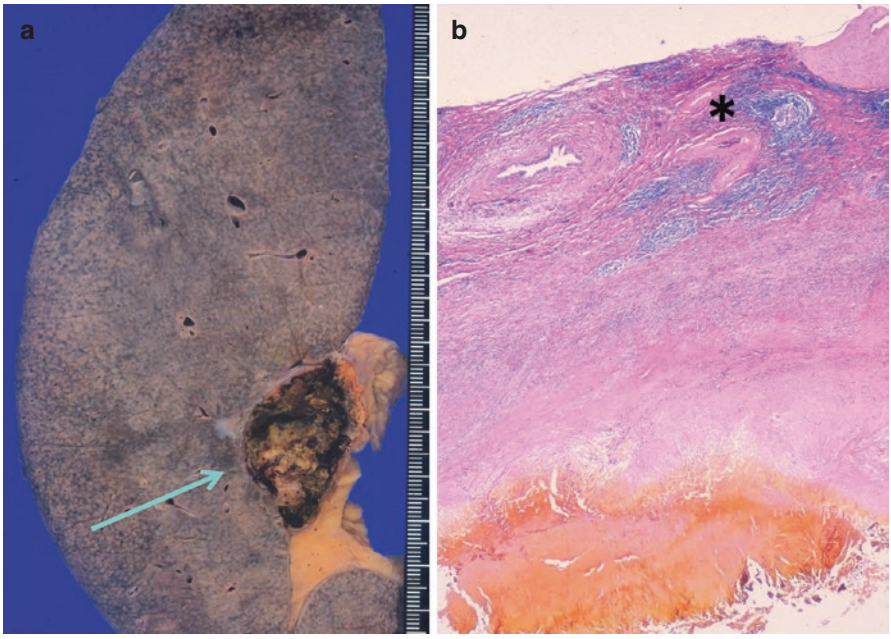


**Fig. 5.5** Biloma. (**a**) An encapsulated collection of bile in the hilum of the liver. (**b**) Extravasated bile (*) is surrounded by granulation tissue and surrounding liver parenchyma. H&E., x60 (original magnification)

#### 5.3.3.2 Bile Leak and Biloma

Biloma, a secondary encapsulated collection of bile in the space of the extrabiliary location, is generally caused by injury to the biliary tree due to trauma or surgery (Fig. 5.5a) [18]. However, spontaneous biloma can be associated with other biliary diseases such acute cholecystitis. Biloma induced by TACE seems to be a consequence of occlusion of small peripheral hepatic arteries with lipiodol, followed by BDN and the leak of bile followed by the formation of biloma. Subsequently, the granulation tissues grow into the area and the resultant fibrous tissue limits the extent of the biloma (Fig. 5.5b).

#### 5.3.3.3 Biliary Strictures Including Sclerosing Cholangitis

The benign strictures and sclerosing cholangitis of biliary tree occur as a complication of biliary ischemia such as LT and TACE (Fig. 5.2b). The latter is characterized by multiple strictures and beaded appearance of the biliary tree [6, 18]. The pathologic features of a narrowed bile duct are intense fibrosis and granulation tissue associated with denudation of epithelial layer; however, there are relatively few signs of inflammation in the bile ducts.

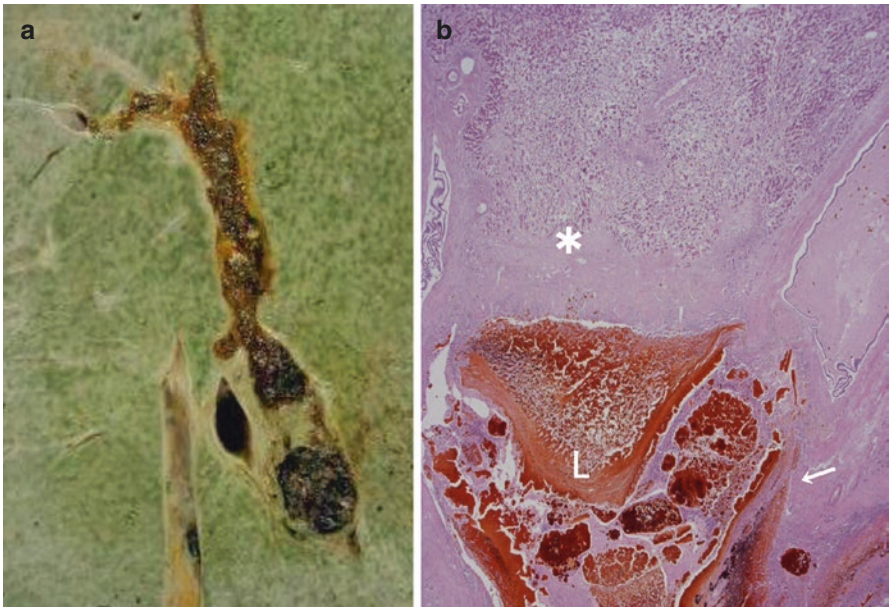


**Fig. 5.6** Progressive sclerosing cholangitis and biliary casts after septic shock. (**a**) The intrahepatic large bile ducts are dilated, and their walls are fibrously thickened and imbibed by bile. *Dark brown*-colored casts in the bile duct lumen are attached to the bile duct walls. Background liver is cholestatic. Autopsy liver. (**b**) Large bile duct shows bile duct fibrosis, necrosis (*arrow*) with bile imbibition, and casts (*) and sludge. *L* bile duct lumen. H&E., x70

#### 5.3.3.4 Bile Duct Loss

Bile duct loss, particularly small bile duct loss, occurs as a biliary ischemia such as chronic rejection. This effect is thought to be modulated via the arteriopathy accompanying chronic rejection with narrowing of the medium-sized arteries. Reduced blood flow of PVP with reduced PVP density around small bile ducts is responsible for the loss of small bile ducts characterizing chronic rejection.

#### 5.3.3.5 Biliary Casts

Biliary casts develop in biliary cast syndrome in which biliary hypoxia plays an important role (Fig. 5.6). Cast materials which are soft or hard and may adhere to the bile duct wall are composed of necrotic material of the intrahepatic and extrahepatic bile duct.

## 5.4 Ischemic Cholangiopathy

The followings are representative ischemic cholangiopathies which are well known to develop in LT and also in interventional approaches such as TACE.

### *5.4.1 Ischemic Cholangiopathy in LT*

There are a variety of intractable disorders that can occur after LT, the most frequent being bile duct strictures and biliary cast syndrome.

#### 5.4.1.1 Biliary Strictures

Bile duct strictures are grouped into anastomotic biliary stricture (AS) and nonanastomotic biliary strictures (NAS) with a reported incidence of anastomotic strictures varying between 1% and 15% and of NAS between 5% and 30% after LT [7, 13].

Anastomotic Biliary Strictures

ASs are isolated strictures at the site of the bile duct anastomosis, and they result mainly from surgical technique and local ischemia, leading to fibrotic scarring of the anastomosis [1, 4].

Nonanastomotic Ischemic-Type Biliary Lesions (ITBLs)

While NASs were first described in LT associated with hepatic artery thrombosis, NASs also occur more frequently with an open hepatic artery and the typical cholangiographic features characterized by strictures and dilatations of one or more large bile ducts on a cholangiogram [1, 4]. At present, this complication in the absence of hepatic artery thrombosis has been designated as nonanastomotic ischemic-type biliary lesions (ITBLs). While NAS may occur in the extrahepatic or intrahepatic biliary tree of the graft, the large intrahepatic and hilar bile ducts are common affected sites. The three-layer organization of PVP is well developed in these biliary segments, and this may be more vulnerable to ischemic injuries [3, 5, 19]. Most ITBLs occur within 1 year of LT [4]. The unique anatomy of the bile ducts supplied by PVP expressing graft-specific antigens targeted by donor-specific antibodies is one of crucial pathogenesis for ITBLs.

Pathogenesis of ITBLs

Although the pathogenesis of ITBLs is multifactorial, there are three main interconnected mechanisms responsible for their formation: ischemic injuries, bile salt-induced cytotoxic damages, and immunological-mediated injury.

*Microangiopathic Injuries to the PVP*

Microangiopathic injuries to endothelial cells of PVP (preservation-induced injury, and prolonged cold and warm ischemia) may be responsible for ischemic injuries to biliary epithelia followed by the development of ITBLs (Fig. 5.3). Cold and warm

ischemic insult can induce direct injury to the BECs, which in turn leads to apoptosis and necrosis of BECs. Particularly, H/R injuries during LT may result in endothelial cell activation and trigger a cascade of events leading to microvascular thrombosis, microcirculatory disturbances, and ischemia of bile ducts [13, 20].

Hypoxic damages on the endothelial cells of PVP lead to subintimal edema and subsequently cause arteriolonecrosis which may also participate in the ischemic injuries of PVP [7].

*Immunopathologic Injuries to the PVP and Biliary Epithelial Injuries*

Immunological injury is assumed to be a risk factor based on the relationship of ITBL.

**ABO incompatibility**: The incidence of ITBLs after ABO-incompatible LT is reportedly higher than after ABO-compatible LT [4, 11]. Persisting ABH antigen expression on BECs after ABO-incompatible LT may be responsible for the occurrence of ITBLs because BECs of the graft bile duct are the target of isoagglutinin (IA) attack [4]. In addition, endothelial injuries in the microvasculature supplying the graft bile duct, which can result from IA attack on the graft vascular endothelium expressing ABH antigens, often cause ischemic biliary epithelial injuries that are followed by the development of ITLBs [16].

**Class I and II HLA antibodies**: HLA class I and II expression in endothelial cells of PVP and BECs is also an immunologic characteristic of these cells in both normal and pathologic conditions. The PVP is thought to be a target for injury by anti-class I and II HLA antibodies. Recent studies suggest that HLA class I and II DSAs against endothelial cells of the PVP and/or cholangiocytes may cause endothelial injury of the PVP, followed by ischemia in BECs, and may result in ITLBs [21]. Kaneku et al. found that 8.1% of LT patients developed de novo DSAs 1 year after LT and demonstrated that de novo DSA development after liver transplantation is an independent risk factor for patient death and graft loss [20].

*Bile Acid Toxicity*

In ITBLs, ischemia due to microvascular damage of the PVP may also lead to impaired biliary $HCO_3^-$ secretion by BECs, followed by uncontrolled permeation and damage from hydrophobic cytotoxic bile acids and resultant biliary epithelial injuries [14, 16].

*Roles of Peribiliary Gland Injuries*

The observation that biliary injury is almost universally present at the time of transplantation, biliary strictures are seen in only a minority of transplant recipients, raising a possibility that proliferation and regeneration of the bile duct wall and epithelium, rather than the initial injury alone, are important determinants in the pathogenesis of biliary strictures [8]. Peribiliary glands of large bile ducts have been identified as a niche of biliary tract stem cells that contribute to regeneration of biliary epithelium after injury, and peribiliary glands are supplied by PVP [8, 22]. After LT, insufficient

regeneration and repair capacity of biliary epithelia related to ischemia may be involved in the development of ITBLs [2, 13, 14]. Op den Dries et al. [8] reported that injuries of extramural peribiliary glands were more prevalent and more severe in livers that later developed ITBLs. In parallel, injury of the PVP was more severe in livers that developed ITBLs. Injuries of PVP before LT are strongly associated with the occurrence of ITBLs. These findings suggest that insufficient regeneration due to loss of peribiliary glands due to ischemic injuries which are related to endothelial injuries of PVP supplying peribiliary glands caused by microangiopathic as well as immunopathologic injuries as mentioned above may explain the development of ITBLs.

#### 5.4.1.2 Biliary Cast Syndrome

Biliary cast syndrome (BCS), which is defined as the presence of cast(s) within the biliary system, is identified by typical cholangiographic findings such as contrast material filling defects compatible with the presence of biliary casts [4, 22]. Fiber choledochoscope examination reveals that the biliary tract is filled with solid substances. BCS is reported as a biliary complication of LT, with the incidence between 3% and 18% [4, 22]. Biliary casts usually form within the first 2 years after LT and lead to bile duct obstruction and biliary infection and result in severe jaundice with subsequent severe liver damages. BCS is also known to develop in non-liver transplant patients.

Biliary casts can be soft or hard and may adhere to the bile duct wall and appear as columnar, antler, leafless tree or dendritic shapes within the bile duct and are composed of necrotic material of the intrahepatic and extrahepatic bile duct (Fig. 5.6) [18]. The immediately removed biliary casts vary in color (black, dark brown, and light brown). The pathological changes in biliary casts include the deposition of bilirubin pigments or crystals with hyperplastic fibrous tissue (bile duct wall), presence of BECs, and infiltrating inflammatory cells in cast surfaces. The presence of small blood vessels and collagen fibers in the biliary casts is related to injury of the bile duct mucosa and wall.

Pathogenetically, BCS is reported to develop in the setting of hepatic/biliary ischemia and biliary strictures. In fact, ITBL and also ASs are frequently associated with BCS after LT [18]. In addition, the occurrence of BCS is also causally associated with biliary obstruction, increased bile viscosity, acute and chronic rejection, infection, bile stasis, alteration of bile metabolism, cold and warm ischemia episodes, and reperfusion injury [4, 18]. Ischemia due to microvascular damage of the PVP may lead to impaired biliary $HCO_3^-$ secretion by BECs, and this may be followed by biliary epithelial injuries and then BCS [14].

### *5.4.2 Ischemic Cholangiopathy in TACE*

Ischemic biliary complications such as biloma, BDN, and focal strictures of large bile ducts are not uncommon in patients treated with TACE [5, 6]. Diffuse dilatation of the intrahepatic bile ducts after TACE may be secondary to these ischemic biliary

injuries. Several possible mechanisms can be involved in the development of such ischemic injuries of the bile ducts after TACE. Gelfoam, lipiodol, chemotherapeutic agents, or mechanical vascular injuries during the procedure could induce the thrombosis or embolization of the arteries and their branches including PVP with inflammation and interrupt the blood supply to the bile ducts followed by the above-mentioned ischemic biliary injuries [6]. Gelfoam can obliterate the larger arteries, whereas smaller particles of lipiodol embolize only smaller arterial branches. The hilar bile duct is even more prone to ischemia from hepatic artery embolization as the middle and distal segments of the CBD have some collateral circulation from the gastroduodenal and posterior duodenal arteries.

#### 5.4.2.1 Focal Biliary Strictures and Sclerosing Cholangitis

Focal stricture of large bile ducts develop after repeated ischemia for prolonged periods by embolization with Gelfoam and repeated direct mechanical injuries to vessel walls caused by the catheter. The pathologic features of a narrowed bile duct after repeated TACE using Gelfoam are intense fibrosis associated with few signs of inflammation in the bile ducts. TACE is followed by a form of sclerosing cholangitis characterized by beaded appearance of biliary tree with multiple strictures, often associated with thrombotic occlusion and/or considerable reduction of PVP, and is reported after TACE with hepatic arterial infusion of chemotherapeutic agent, such as floxuridine or bleomycin [6].

#### 5.4.2.2 Biloma and Bile Duct Necrosis

BDN after TACE, particularly the peripheral BDN, is well known, and US examination for dilation of the bile duct in the liver is able to confirm the diagnosis of BDN. In addition, cholangiography images showing marked stenosis of the hepatic duct may also indicate BDN.

Biloma has been reported after TACE or direct injuries of the biliary tree by surgery or trauma. Biloma formation induced after TACE seems to be a consequence of BDN and leak of bile due to the occlusion of small peripheral hepatic arteries with lipiodol. In a postmortem study of patients with HCC treated with TACE, about one half of the nonnecrotic bile ducts adjacent to the necrotic bile ducts of bilomas demonstrated marked reduction of the inner layer of vessels of PVP [5].

### *5.4.3 Progressive Sclerosing Cholangitis After Septic Shock*

This type of cholangitis is causally associated with burn injury, polytrauma, extensive surgery, or sepsis and is a recently increasingly diagnosed disease entity [13, 23]. The diagnosis of sclerosing cholangitis is based on cholangiography. The bile

ducts were partially filled by black-pigmented or necrotic material (biliary cast) (Fig. 5.6). The clinical course varies considerably; liver disease rapidly progresses to cirrhosis in most cases, while the others present with slower progression and with recurrent episodes of cholangitis.

As for the pathogenesis, endothelial injuries by direct toxic effects of bacterial endotoxins and lipopolysaccharides (LPS) on microvascular endothelium lead to endothelial injuries in sepsis or endotoxemia. Furthermore, LPS-induced massive release of proinflammatory cytokines from PVP can cause destruction of the mucosa and wall of bile ducts. Neutrophils, adherent to the endothelial surface, cause increased microvascular permeability and local edema. Gut barrier failure follows and may induce translocation of bacteria and endotoxins from the intestinal lumen into the portal blood and possibly into the microvasculatures of bile ducts. Following cholangitis, postinflammatory biliary strictures may develop. Moreover, thermal injuries in particular can induce selective vasoconstriction of hepatic arterial blood flow. This additional hepatic ischemia and critical reduction of hepatic oxygen delivery may lead to sclerosing cholangitis.

## 5.5 Conclusion

Ischemic cholangiopathies are composed of several clinicopathological categories due to different etiopathogeneses, though they share several pathological features such as bile duct sclerosis or strictures and BDN. Ischemic cholangiopathies related to liver transplantation and also those associated with TACE are well known for the development of ischemic cholangiopathy such as biliary strictures and necrosis, though ischemic cholangiopathies also occur spontaneously and in other medical treatments. Endothelial damages of PVP due to antibody-mediated attack microangiopathic injuries may also lead to ischemic cholangiopathies. In addition, ABH blood type and HLA antigens expressing on endothelial cells in the PVP and BECs in the large bile ducts could be targets by an immunological attack. Further studies on the PVP and bile ducts from anatomical and immunological aspects are mandatory for evaluation of and therapeutical approach to ischemic cholangiopathies.

**Disclosures** None

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# Part 2
# Practical Understanding of Biliary Diseases

# Chapter 6
# Immunopathology of Bile Duct Lesions of Primary Biliary Cirrhosis

**Hayato Baba, Ayumi Sugitani, Ryusei Takahashi, Kouki Kai, Yuki Moritoki, Kentaro Kikuchi, and Koichi Tsuneyama**

**Abstract** Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease that predominantly affects women and is characterized by chronic progressive destruction of the small intrahepatic bile ducts with portal inflammation and ultimately fibrosis. The serologic hallmark of PBC is the presence of anti-mitochondrial autoantibodies (AMA). Several mechanisms may now be proposed regarding the immune-mediated bile duct damage in PBC, including the possible roles of T cells, B cells, AMA, and other cell phenotypes. Weakness of biliary epithelial cells in association with apoptosis, senescence, and autophagy has also been noted recently. In PBC, several complex steps and mechanisms may be involved in the induction and progression of cholangitis and biliary degeneration, followed by bile duct loss.

**Keywords** Anti-mitochondrial autoantibodies (AMA) • Immune-mediated bile duct damage • Biliary epithelial degeneration

## 6.1 Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by chronic nonsuppurative destructive cholangitis (CNSDC) associated with selective destruction of the intrahepatic small bile ducts (interlobular bile ducts and septal bile ducts) by inflammatory cells, mainly lymphocytes and plasma cells. The

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appearance of anti-mitochondrial autoantibodies (AMA) (90–95%) and a high serum IgM level are characteristics of PBC patients' sera [1, 2]. Progressive biliary damage eventually causes bile duct loss and fibrosis. The mechanisms by which such antibodies produce liver tissue injury are still unknown; however, exposure to infectious microbes and/or xenobiotics is conjectured to initiate an immune reaction stemming from an individual's genetic predisposition and the participation of innate immunity at the primary stage of PBC.

As the mechanism of biliary damage, immunological interaction between biliary epithelial cells and surrounding inflammatory cells is important. It is considered that biliary epithelial cells in PBC express various cytokines and chemokines to generate and continue the specific inflammatory condition around them. Damaged biliary epithelial cells have an antigen-presenting ability, with aberrant expression of HLA class II and other co-stimulatory molecules [3, 4]. Various kinds of migratory inflammatory cells become effector cells, which attack biliary epithelial cells and also produce additional cytokines and chemokines to produce a subsequent inflammatory status and progressive fibrosis. Biliary weakness, showing cellular senescence and disturbance of autophagy, accelerates biliary destruction. This report reviews the immunopathological characteristics of injured bile ducts and infiltrating effector cells, including T cells, natural killer (NK) cells, and B cells, in PBC.

## 6.2 Immunopathological Characteristics of Damaged Biliary Epithelial Cells and Surrounding Inflammatory Cells in PBC

The small bile ducts, not the large bile ducts, are the specific targets of PBC [5]. Chronic nonsuppurative destructive cholangitis (CNSDC) is one of the typical images of the small portal tracts in PBC patients (Fig. 6.1a). Infiltrating inflammatory cells invade the epithelium and cause epithelial interruption and ductal luminal irregularity. Biliary epithelial cells become fragmented and finally disappear (ductopenia, bile duct loss). Lymphocytes, plasma cells, and often eosinophils infiltrate the areas around the damaged bile ducts. Epithelioid granulomas in various sizes constructed by aggregated macrophages frequently appear in the portal area and in the hepatic parenchyma (Fig. 6.1b).

Biliary epithelial cells of PBC aberrantly express various kinds of co-stimulatory factors and adhesion molecules as well as major histocompatibility complex (MHC) class II molecules and may express the target molecules [3, 4, 6]. Biliary epithelial cells themselves also express various kinds of cytokines/chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), RANTES (regulated on activation, normal T cell expressed and secreted), fractalkine, as well as generating specific immunological microenvironments around them [7–12]. Biliary epithelial cells also bear several cytokine receptors against IL-4, IL-6, interferon-gamma (IFN-γ), and TNF-α, so these cytokines have autocrine and paracrine effects [13].

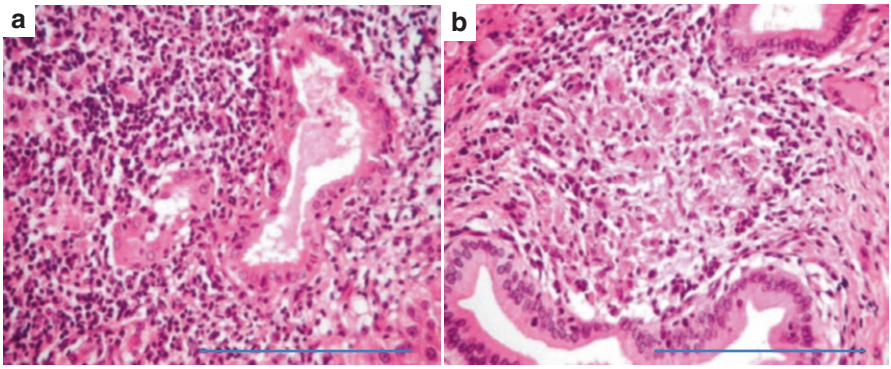


**Fig. 6.1** Typical images of chronic nonsuppurative destructive cholangitis (CNSDC) (**a**) and epithelioid granuloma near a damaged bile duct (**b**) in a patient with primary biliary cirrhosis (PBC) (scale bar, 100 μm)

Various studies have described the profiles of the inflammatory cells around the bile ducts in PBC [9, 14–17]. It is considered that the cellular profile may change depending on the process of biliary inflammation. Different immune cell profiles are frequently observed at different portal areas of the same hepatic tissue section. However, it is generally believed that T-cell-related cellular immunity is involved in the pathogenesis of CNSDC. Indeed, it has been demonstrated that CD8+ and CD4+ lymphocytes are the predominant cell types in the inflammatory cells within the portal area in PBC [18, 19]. CD8+ lymphocytes are mostly cytotoxic T cells and affect the targets via the perforin/granzyme exocytosis pathway [20, 21]. CD4+ lymphocytes, especially pathogenic autoreactive T cells, regulate autoimmunity around the bile ducts in PBC. In the early stage, a Th1-predominant cytokine milieu is characteristic in CNSDC [22]; however, as the disease progresses, both IL-12/Th1 cells and IL-23/Th17 cells appear in various degrees. In the late stage of PBC, the ratio of cell formation shifts from Th1 cells to Th17 cells [16]. A decreased number of portal-infiltrating regulatory T cells and an imbalanced ratio with cytotoxic T cells may be associated with disease progression [23, 15].

While T cells comprise 55% of the cellular infiltrate, macrophages make up about 30% [24], and B cells/plasma cells account for about 10% of the population [19, 25]. Eosinophils may make up some proportion of the inflammatory cells [26, 27, 9]. Natural killer (NK) cells and NK T cells account for approximately 5% of the cellular infiltrate and play important roles in initiating the breakdown of tolerance [28]. Interdigitating dendritic cells are found between the biliary epithelial cells, often near breaks in the basement membrane and in the periductal granulomatous response [4, 29, 30].

Lymph follicles are frequently observed in the portal tracts of PBC patients, both in the early stage and the late stage [31] (Fig. 6.2). In immunohistochemical analysis, lymph follicles of PBC are considered as tertiary lymphoid organs (TLOs) (Fig. 6.3). Briefly, high endothelial venules (HEV) showing MECA-79 expression are located in the center of the lymph follicles. A follicular dendritic cell (FDC)

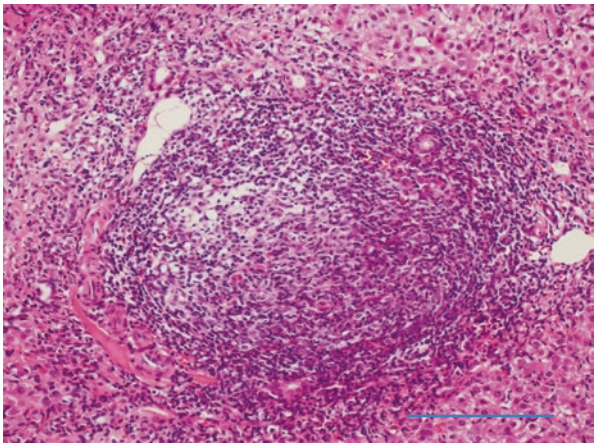

**Fig. 6.2** Lymph follicle formation with germinal center in a portal tract of a patient with primary biliary cirrhosis (PBC) (scale bar, 100 μm)

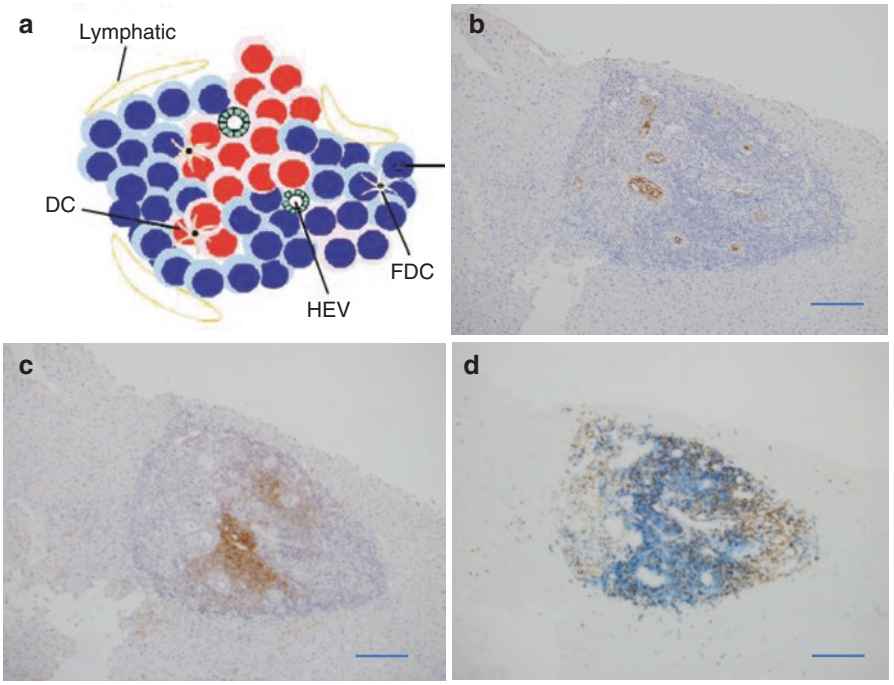


**Fig. 6.3** (**a**) A schema of tertiary lymphoid organs (TLOs) in a portal tract of a patient with PBC (*DC* interdigitating dendritic cells, *HEV* high endothelial venules, *FDC* follicular dendritic cells). (**b**) Immunostaining of MECA-79, which is a marker of high endothelial venules (HEV) (*brown color*, positive; counterstaining was performed by hematoxylin). (**c**) Immunostaining of CD21, which is a marker of follicular dendritic cells (*brown color*, positive; counterstaining was performed by hematoxylin). (**d**) Double immunostaining of CD3, which is a marker of T cells and CD20, which is a marker of B cells (*brown color*, CD3; *blue color*, CD20) (scale bar, 100 μm)

network with CD21 expression is observed around the HEV. B cells are seen to be regularly accumulated along the FDC network and located in the center part of the lymph follicles. A layer of T cells is observed in the outer part of the B-cell layer. In our unpublished data, 78% of PBC cases with inflammatory cell infiltration had MECA-79-positive HEV formation. A continuous inflammatory process may induce MECA-79-positive HEV initially and cause subsequent TLO formation in the portal area. These TLOs often include injured bile ducts; however, TLOs formed far away from the injured bile ducts have also been observed. Any direct correlation between TLOs and injured bile ducts is still unknown. Takahashi et al. reported that, rather than follicle-like aggregation of CD20-positive B cells, periductal infiltration of CD38-positive plasma cells is highly associated with bile duct injury [32]. TLOs may correlate indirectly with bile duct injury via maturation of effector cells.

## 6.3 Mechanism of Bile Duct Injury in PBC

There are several pathogenetic possibilities for the mechanism of bile duct injury in PBC. One hypothesis for the selective destruction of biliary epithelial cells is that the pyruvate dehydrogenase complex (PDC)-E2 subunit, which is normally located in the mitochondrial inner membrane, is aberrantly expressed on the surface of biliary epithelial cells [3]. Not only PDC-E2 itself but also its mutant form and tissue-specific variants may cause the same phenomenon. Lleo et al. reported the existence of intact immunoreactive PDC-E2 within apoptotic blebs of cholangiocytes during the process of apoptosis in PBC [33, 34]. An autoimmune response may accelerate the process against these modified intrinsic PDC-E2 or related molecules.

Another hypothesis is the occurrence of an immune reaction against extrinsic antigen located in the biliary epithelium. The microbial mechanism termed "molecular mimicry" is a strong hypothesis being advanced to account for the breaking of tolerance against mitochondrial antigens.

Epidemiological studies have also suggested that infectious agents can trigger or even exacerbate the disease [35]. Both gram-positive and gram-negative bacteria have been suspected, especially *Escherichia coli* and *Novosphingobium aromaticivorans*, which are the most commonly associated agents that have been reported to date [36–38]. Another candidate for the role of extrinsic antigen is xenobiotics (chemicals). Many chemicals, including pharmaceuticals and household detergents, have the potential to form metabolites that show molecular mimicry to PDC-E2 [39, 40]. Amano et al. reported that 2-octynoic acid was unique in both its quantitative structure-activity relationship analysis and reactivity. Sera from PBC patients demonstrate high Ig reactivity against 2-octynoic acid-PDC-E2 peptide. Not only does 2-octynoic acid have the potential to modify PDC-E2 in vivo, but, importantly, it is widely used in the environment including in perfumes, lipstick, and many common food flavorings [41]. Mice immunized with 2-octynoic acid serve as a unique PBC animal model showing autoimmune cholangitis, typical anti-mitochondrial autoantibodies, and increased number of liver lymphoid cells with an increase in the number of CD8(+) cells in the liver [42].

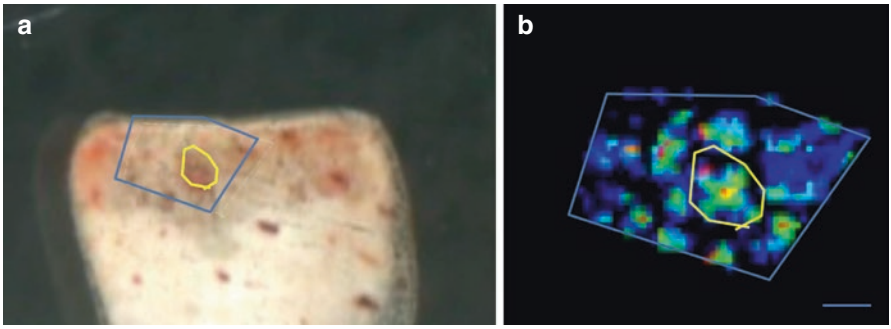


**Fig. 6.4** Localization of 2-octynoic acid on the liver of model PBC mice (frequent abdominal injections of 2-octynoid acid). (**a**) Image of a frozen section. *Blue* rounded area was analyzed by imaging mass spectrometry using the nanoparticle-assisted laser desorption/ionization (nano-PALDI) method. The *yellow* rounded area is the portal area. (**b**) Enlarged image of nano-PALDI mass spectrometry of *blue* rounded area. Not only the hepatic parenchyma but also the portal area has positive signals of 2-octynoic acid (scale bar, 100 μm)

However, demonstrating the localization of 2-octynoic acid in the liver has been difficult because of its small molecular size. We have recently developed a new technique to highlight the expression of low-molecular-weight molecules without any labeling on frozen liver using nanoparticle-assisted laser desorption/ionization (nano-PALDI) imaging mass spectrometry (IMS) [43]. We examined the localization of 2-octynoic acid in the liver by using 2-octynoic acid-induced PBC model mice (Fig. 6.4). Interestingly, 2-octynoic acid was not only located in the hepatic parenchyma but also in the portal area. Because 2-octynoic acid was also detected in bile juice, we speculated that 2-octynoic acid may deposit in biliary epithelial cells. These results imply that aberrantly deposited extrinsic xenobiotics (chemicals) or their metabolites can act as pathogens. We are planning to examine frozen liver samples from PBC patients to clarify the pathogenic roles of various xenobiotics.

The activation of the innate immune response seems to be another key event in early PBC that leads to autoimmune injury of the small intrahepatic bile ducts. Biliary epithelial cells possess an innate immune system consisting of the Toll-like receptor (TLR) family, which recognizes pathogen-associated molecular patterns (PAMPs). In PBC, deregulated biliary innate immunity, namely, hyperresponsiveness to PAMPs, is associated with the pathogenesis of cholangiopathy. Moreover, the targeted biliary epithelial cells may play an active role in the perpetuation of autoimmunity by attracting immune cells via chemokine secretion. Biliary innate immune responses induce the production of two chemokines, fractalkine and several Th1 shift chemokines, causing the migration of inflammatory cells including NK cells. TLR4 ligand-stimulated NK cells destroy autologous biliary epithelial cells in the presence of IFN-α synthesized by TLR3 ligand-stimulated monocytes. These findings give new insights into the pathogenesis of PBC [44, 45].

Injured bile ducts and bile ductules of PBC indicate a cellular senescence. Senescent biliary epithelial cells can modulate the microenvironment around bile ducts by expressing senescence-associated secretory phenotypes (SASP) and contribute to maintaining inflammation and fibrosis around bile duct lesions in PBC. Deregulated autophagy followed by cellular senescence in biliary epithelial cells may be closely related to the abnormal expression of mitochondrial antigens and subsequent autoimmune pathogenesis in PBC [46, 47]. Biliary epithelial cells of PBC suffer strong oxidative stress because of a decrease in their antioxidative ability [48]. Oxidative stress may accelerate cellular senescence and disturbance of the autophagy system, causing complex biliary damage.

## 6.4 Relationship Between the Manifestation of AMA and Bile Duct Injury

Various ideas have been offered regarding the participation of AMA in bile duct injury; the author recently suggested a protective contribution of AMA against biliary damage [31]. The degree of bile duct damage around the portal areas was significantly milder in AMA(+) PBC than that observed in AMA(-) PBC in liver biopsy examination of Chinese PBC patients [31]. Conversely, Lleo et al. suggested that AMA promotes the inflammatory process by demonstrating that there is intense inflammatory cytokine production in the presence of biliary epithelial cell apoptopes, macrophages from patients with PBC, and AMA. The cytokine secretion was inhibited by anti-CD16 and was not due to differences in apoptope uptake. Moreover, mature monocyte-derived macrophages from PBC patients cultured with biliary epithelial cell apoptotic bodies in the presence of AMA markedly increased tumor necrosis factor-related apoptosis-inducing ligand expression [33].

Several reports on AMA in animal models have also been published [23]. A unique murine PBC model expressing a dominant negative form of transforming growth factor beta receptor II (dnTGFbetaRII) under control of the CD4 promoter developed both colitis and autoimmune cholangitis with elevated serum levels of IL-6. Based on this observation, IL-6-deficient mice with a dnTGFbetaRII background (dnTGFbetaRII IL-6(-/-)) were produced and examined for the presence of AMA, cytokine levels, histopathology, and hepatic immunohistochemistry. Serum AMA levels decreased in the dnTGFbetaRII IL-6(-/-) mice; however, autoimmune cholangitis was significantly exacerbated, including elevated levels of inflammatory cytokines, increased number of activated T cells, and worsening hepatic pathology. These results suggest an inflammatory inhibitory action of AMA.

To inhibit AMA secretion, autoreactive B-cell depletion therapy using several PBC model mice was also performed. Moritoki et al. examined the therapeutic

efficacy of B-cell depletion using anti-CD20 [49]. In mice whose treatment was initiated at 4–6 weeks of age (early treatment group), anti-CD20 therapy demonstrated a significantly lower incidence of liver inflammation associated with reduced number of activated hepatic CD8(+) T cells. In contrast, in mice treated at 20–22 weeks of age (late treatment group), anti-CD20 therapy had relatively little effect on the liver. All treated animals had reduced levels of B cells, absence of AMA, and increased levels of inflammatory cytokines such as TNF-α in sera. AMA may play some roles for the induction state of pathogenesis, but not for disease progression in this model [49]. However, B-cell depletion using another murine PBC model (genetic B-cell-deficient Igmu(-/-) NOD.c3c4 mice) demonstrated reduced levels of B cells, absence of AMA, and a decreased number of non-B cells in the liver accompanied by reduced number of activated NK cells. Since liver inflammation was significantly attenuated, B cells and AMA may play important roles in pathogenesis in the model [31]. Given the disparate nature of these results, we consider that the role of B cells and AMA may depend on the disease phase and a variety of other factors.

## 6.5 Hyper-IgM Production and Immunopathology

Elevated levels of IgM and the presence of AMA are characteristic of the sera of PBC patients. The increase in serum IgM is considered to be the result of chronic B-cell activation induced via the TLR-signaling pathway. Indeed, peripheral blood mononuclear cells (PBMCs) from PBC patients produce significantly higher levels of polyclonal IgM and secretion of AMA than controls after exposure to CpG, which is a natural ligand for TLR9 [50, 51].

The primary site of IgM production in PBC patients is still unclear. Takahashi et al. reported that CD38-positive plasma cells accumulated around the bile duct in PBC patients [32]. These periductal plasma cells produced IgM and IgG, not IgA, so they may be candidates for the source of serum IgM. Kikuchi et al. focused on the spleens of PBC patients, because B-cell maturation and differentiation occur in the splenic white pulp and produce IgM in response to an innate immunity stimulus of capsular polysaccharide of a pneumococcus. In immunohistochemical analysis using surgically resected spleens and autopsied spleens, IgM-producing plasma cells aggregated near the CD21-positive FDC network into the germinal center of the spleens of PBC patients (Fig. 6.5). A chemokine, CXCL13, which has a chemotactic function for B cells, localized near IgM into the lymph follicles of PBC spleens. Not only portal-infiltrated B cells in the liver but also splenic B cells produced IgM in the PBC patients. In PBC patients, IgM production may be regulated systemically, rather than being a local event in the liver.

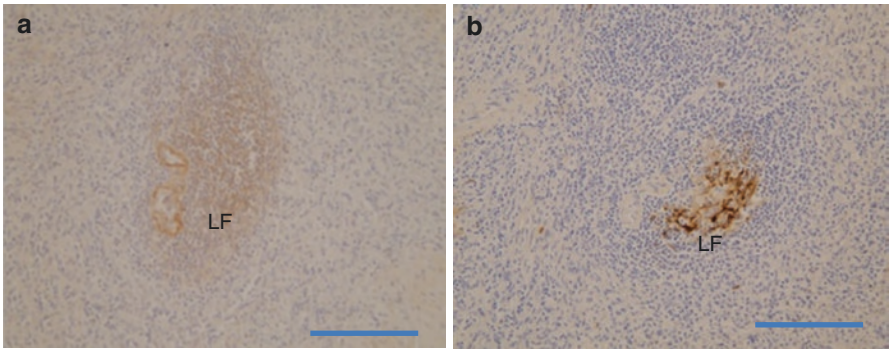


**Fig. 6.5** Immunostaining of the lymph follicle of the spleen in patients with primary biliary cirrhosis (PBC). (**a**) CD21 immunostaining showing the follicular dendritic cell (FDC) network. (**b**) IgM immunostaining showing aggregation into the germinal center (*LF* lymph follicle; scale bar, 100 μm)

## 6.6 Establishment of a New Therapeutic Approach Against B Cells in PBC

A new therapeutic approach targeting B cells in PBC patients has recently been clinically performed. Tsuda et al. reported the safe and potential efficacy of B-cell depletion with the anti-CD20 monoclonal antibody rituximab in patients with PBC who had experienced incomplete response to ursodeoxycholic acid (UDCA) [52]. After treatment, serum levels of total IgG, IgM, and IgA as well as AMA-IgA and AMA-IgM decreased significantly from baseline by 16 weeks and returned to baseline levels by 36 weeks. Transient decreases in memory B cells and T cells and an increase in CD25(high) CD4(+) T cells were observed after treatment. These changes were associated with significant increases in mRNA levels of FoxP3 and TGF-β and a decrease in TNF-α in CD4(+) T cells. Notably, serum alkaline phosphatase levels were significantly reduced up to 36 weeks following rituximab treatment. From the above results, Tsuda et al. concluded that depletion of B cells influences the induction, maintenance, and activation of both B cells and T cells and provides a potential mechanism for treatment of patients with PBC who experience an incomplete response to UDCA. However, in a few examples of PBC patients administered rituximab, the potential for herpes zoster reactivation and upper respiratory infection were acknowledged in this trial. This is regarded as an important problem of infection control in the performance of B-cell removal therapy. We have also experienced a case of PBC that rapidly progressed to liver cirrhosis after treatment including rituximab [53]. A 66-year-old Japanese female patient with PBC, who presented with a gastric lymphoma, had been treated with a regimen containing rituximab for incidental malignant lymphoma. She showed biochemical and

immunological improvements, and her liver histology before and after rituximab treatment confirmed a decrease in liver inflammation. However, she developed liver cirrhosis a short time after rituximab treatment without biochemical or immunological worsening.

In conclusion, autoimmune B-cell removal therapy has the potential to become a new treatment for PBC, but caution should be exercised, and careful patient observation is required.

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# Chapter 7
# Recurrent Primary Sclerosing Cholangitis in Comparison with Native Primary Sclerosing Cholangitis

**Aya Miyagawa-Hayashino and Hironori Haga**

**Abstract** This chapter covers primary sclerosing cholangitis (PSC) and its overlap with autoimmune hepatitis (AIH) and liver transplantation (LT) for PSC. Histologic findings similar to those of PSC are known to occur in liver allografts transplanted for PSC; this is thought to be a recurrence of the original PSC. We mainly focus on characterizing the histologic features of recurrent PSC after LT with an emphasis on the overlap of active hepatitis and hilar xanthogranulomatous cholangitis with respect to outcome. These histologic findings may represent an important and unrecognized cause of progressive deterioration followed by cirrhotic transformation in recurrent PSC after LT.

**Keywords** Liver transplantation • Recurrent of the original disease • Recurrent PSC • Autoimmune hepatitis • Overlapsyndrome

## 7.1 Primary Sclerosing Cholangitis

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by chronic inflammation and periductal fibrosis of the walls of the biliary tree. The typical PSC patient is a 30–40-year-old male with inflammatory bowel disease, but advances in diagnostic imaging have allowed PSC to be diagnosed at all ages including childhood. Primary sclerosing cholangitis usually affects large intrahepatic and/or extrahepatic bile ducts, but in 5% of cases, only small intrahepatic bile ducts are involved; this is called small-duct PSC [1]. A liver biopsy is not

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necessary to diagnose large-duct PSC in the presence of typical cholangiographic findings. Patients who present with clinical features and a liver biopsy compatible with PSC but with a normal cholangiogram are classified with small-duct PSC.

Liver function tests show cholestasis. Elevated serum alkaline phosphatase (ALP) is a characteristic biochemical finding. Antineutrophil antibodies in a perinuclear pattern (pANCA) are found in approximately 80% of PSC patients, although this test lacks sensitivity and specificity [1]. Although the clinical course varies widely, progressive obliteration of the biliary tree eventually leads to biliary cirrhosis, and LT continues to be the only therapeutic option for patients with end-stage PSC [2].

Typical liver histologic findings in PSC are fibro-obliterative bile duct lesions characterized by an "onion-skin"-like periductal fibrosis around medium-sized or larger bile ducts. Epithelial lining cells are seen to be atrophic and degenerative. Fibro-obliterative lesions eventually replace the bile duct with fibrous scarring and inconspicuous inflammation. Small interlobular bile ducts may also be affected and replaced by fibrous scars in addition to the involvement of larger ducts. Involvement solely of small interlobular bile ducts can lead to the diagnosis of the small-duct variant of PSC [1]. Lymphoplasmacytic inflammation involving the hilar bile ducts and intrahepatic bile ducts is variable. Biliary sludge or microstones are deposited in some affected bile ducts. Xanthogranulomatous changes or severe parenchymal necroinflammation with interface hepatitis is usually not prominent [3].

## 7.2 PSC/AIH Overlap Syndromes

Active hepatitis characterized by distinct interface hepatitis with lymphoplasmacytic infiltration and prominent lobular and perivenular necroinflammation, which are characteristic findings for autoimmune hepatitis (AIH), are unusual in PSC alone; such cases are considered as an overlap of PSC with AIH. Overlap of PSC with AIH is more often found in children and adolescents than in adults in the non-transplant setting [1, 4–7]. Overlap of PSC with AIH often presents with autoimmune features including positive autoantibodies and elevated immunoglobulin G (IgG) in addition to histologic features of AIH, and it is treated as AIH. The diagnosis of PSC may become apparent during the follow-up [8]. Therefore, the possibility of PSC should be considered in all children with AIH [9].

There has been an increasing awareness of the existence of patients having features of both conditions called "PSC/AIH overlap." The International Autoimmune Hepatitis Group (IAIHG) [10] scoring system has been widely applied to define "overlap syndromes," but the scoring system was not actually intended for such use. Patients considered to have such a clinical overlap may only achieve a score diagnostic of AIH in a limited number of cases, and the IAIHG scoring system for AIH should not be used to define overlaps. Currently the overlapping features are widely accepted to constitute a variant of the primary disorder, mostly PSC, rather than a separate disease entity as declared by the IAIHG position statement. Although standardized definitions of "overlap syndromes" have not been developed, "PSC with features of AIH" is proposed to be defined from the presence of disproportionately

elevated serum transaminase and/or IgG levels (ALT at least 5x upper limit of normal (ULN) and IgG at least 2x ULN may serve as guidelines) and histologic features of AIH (interface hepatitis) [9, 11].

As some patients with overlapping features appear to benefit from treatment with a combination of ursodeoxycholic acid and immunosuppressants, the IAIHG position statement proposes that in PSC with features of AIH, immunosuppressive treatment should be considered. However, this strategy is not evidence based, because of the low prevalence of overlap syndromes [9].

## 7.3 Recurrent PSC After Liver Transplantation

### *7.3.1 Clinical Features*

After liver transplantation, anastomotic strictures are the most common biliary complication. A variety of potential insults to the graft result in nonanastomotic biliary structuring possibly by biliary tract ischemia including prolonged cold ischemic times, hepatic artery thrombosis/stenosis, small-for-size graft, and antibody-mediated rejection (e.g., preformed anti-donor antibodies and blood group antibodies) [12]. The terminal portion of the donor bile ducts is susceptible to ischemic injury since it is supplied by only one of the three terminal branches of the hepatic artery [13]. Biliary infections may also cause biliary structuring. It is important to differentiate anastomotic strictures from nonanastomotic strictures. Anastomotic strictures are short, circumscribed strictures localized at the anastomosis and are probably caused by ischemia or fibrosis due to suboptimal surgical technique [12]. By contrast, nonanastomotic strictures are localized proximal to the anastomosis. They are usually multiple and of longer length [12]. The frequency of these known causes of biliary complications is of no difference between PSC group and the control group [14]. However, in various studies, a significantly greater incidence of nonanastomotic strictures has been observed to occur in the PSC group after LT in comparison with the control group. Since no other process has been recognized that explains their greater frequency, this condition is thought to be recurrence of the primary disease, PSC, in the graft [15–17]. One of the main factors impairing the long-term outcome after LT for PSC is recurrent disease. PSC recurrence occurs after LT with an incidence of 5%–20% in cadaveric LT [14, 15, 17]; nevertheless, cadaveric LT provides rather excellent long-term graft survival rates (5-year rate 86% and 10-year rate 70%) for patients with end-stage PSC [18]. However, in living donor liver transplantation (LDLT), which is widely performed in Japan, PSC recurrence has been higher with an incidence of nearly 50% in single-center experiences in Tokyo and Kyoto groups [19–21]. The posttransplantation course of patients with recurrent PSC in LDLT has been poor; the 5- and 10-year graft survival rates have been reported as 69% and 40%, respectively [19, 21]. Donor-relatedness is reported to be one of the risk factors in PSC recurrence [22].

HLA-DRB1*08 in Caucasian populations [23] and HLA-DR15 in the Japanese population are reported to be related to recurrence [19, 20].

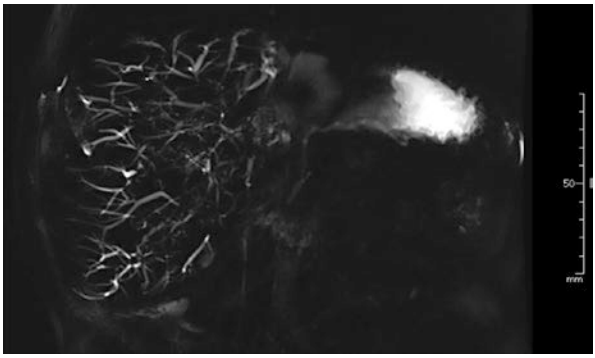

**Fig. 7.1** The diagnosis of primary sclerosing cholangitis (PSC) recurrence after liver transplantation (LT) should be based primarily on cholangiographic findings of multifocal strictures and beading of the intrahepatic and/or extrahepatic bile ducts with compatible cholestatic biochemical abnormalities after exclusion of secondary causes

### *7.3.2 Definition of Recurrent PSC Posttransplantation*

It is widely accepted that recurrent PSC is diagnosed based on the following criteria defined by Graziadei et al. [14, 17, 18]: (1) a confirmed diagnosis of PSC before liver transplantation, (2) a cholangiogram showing nonanastomotic biliary strictures of the intrahepatic and/or extrahepatic biliary tree with beading and irregularity occurring >90 days posttransplantation (Fig. 7.1), or (3) a liver biopsy showing fibrous cholangitis and/or fibro-obliterative lesions with or without ductopenia, biliary fibrosis, or biliary cirrhosis. Other causes of biliary stricture such as hepatic artery thrombosis/stenosis, chronic ductopenic rejection, ABO blood type incompatibility, and anastomotic bile duct strictures related to surgical reconstruction are excluded [17].

### *7.3.3 Histologic Features of PSC Recurrence*

The histologic criteria for recurrent PSC have been strictly defined; fibrous cholangitis and/or fibro-obliterative lesions are mandatory for the diagnosis of recurrent PSC [14, 15, 17]. These histologic findings are consistent with those of nontransplant PSC, and almost all reported series have described the histology of PSC recurrence as compatible with the criteria [18]. Since the characteristic radiologic findings in the proper clinical setting are the confirmatory test for the diagnosis [1, 18], the detailed histologic findings related to outcome for PSC recurrence post-LT have not been well described [19, 24, 25]. We characterized the additional histologic features in the liver allografts after LDLT, of which the description will be made subsequently [3].

There have been a few detailed histologic descriptions concerning the association of active hepatitis with recurrent PSC. Demetris et al. stated that association with active hepatitis is unusual [26], and Khettry et al. observed lymphoplasmacytic lobular hepatitis in one of six definite cases of recurrent PSC [24]. Khettry et al. described mild necroinflammatory changes associated with graft dysfunction in posttransplant biopsies in the absence of recurrent PSC and termed such cases as autoimmune liver disease [24]. While this condition may be included in de novo AIH, for which autoimmune hepatitis-like changes are seen in liver allografts in patients transplanted for end-stage liver disease not caused by a previous autoimmune liver disease [27], its exact clinicopathological features and category remain unspecified [24].

The histologic findings of recurrent PSC are similar to those in the non-transplant setting in that an interlobular bile duct shows a fibro-obliterative lesion with concentric periductal fibrosis (onion-skin appearance) (Figs. 7.2a, b). As the disease progresses, the fibro-obliterative lesion causes replacement of the bile duct by a fibrous scar with minimal inflammation (Fig. 7.2c). However,

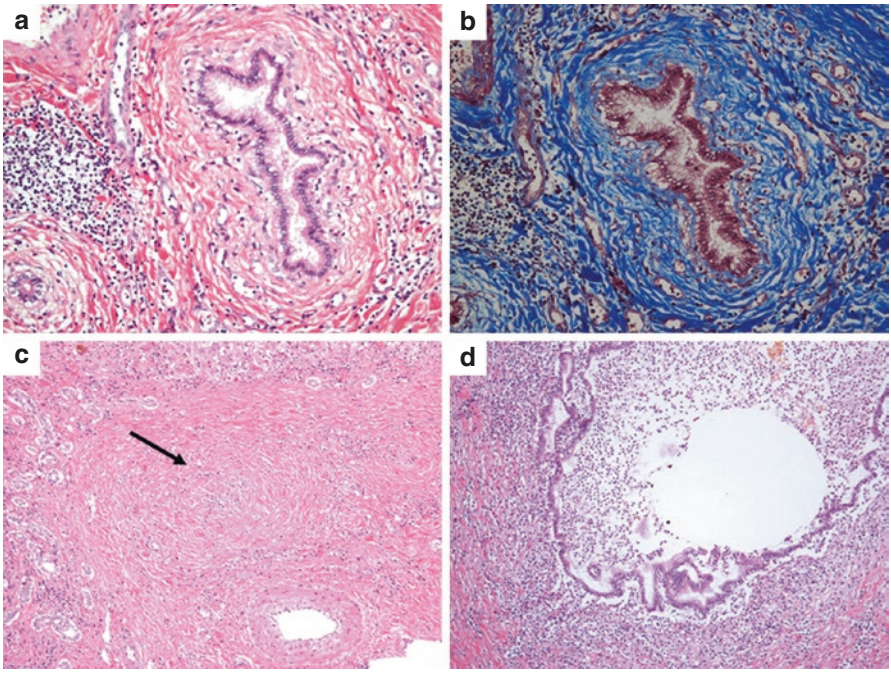


**Fig. 7.2** Explanted liver after living donor liver transplantation is performed for recurrence of primary sclerosing cholangitis (PSC). **a**: An interlobular bile duct shows periductal fibrosis. **b**: Masson trichrome staining of (**a**). **c:** Intrahepatic large bile duct (arrow) shows fibro-obliterative lesions replaced by fibrous scarring with minimal inflammation. **d**: Intrahepatic large bile duct shows xanthogranulomatous change with bile fragment

such characteristic fibro-obliterative lesions are less common in recurrent PSC than in native livers [3]. Instead, patients with recurrent PSC frequently have the additional features of moderate to marked active hepatitis. In particular, there has been a patient with recurrent PSC with active hepatitis who underwent retransplantation, and the explanted liver showed submassive to massive hepatic necrosis [3]. Reportedly, in native livers of patients with AIH/PSC, ductopenia has been relatively mild, and necroinflammation of the hepatic parenchyma has been prominent in comparison with patients who had pure PSC [21].

It may also be found that lymphoplasmacytic cholangitis of the hilar and intrahepatic large bile ducts of recurrent PSC is rather prominent in comparison with native PSC livers. In addition, xanthogranulomatous cholangitis may be evident in half of recurrent PSC livers at the time of retransplantation, although such changes are absent in native livers (Fig. 7.2d) [3]. In a study by Keaveny et al., 16 of 51 native PSC livers demonstrated such xanthogranulomatous cholangitis at the time of liver transplantation, and PSC cases with xanthogranulomatous cholangitis have been associated with a higher rate of early post-LT mortality or retransplantation [25]. These findings suggest that the frequent occurrence of xanthogranulomatous cholangitis in recurrent PSC after LDLT may be at least partly responsible for the rather rapid progression of recurrent PSC compared with the course of native PSC. Although the exact cause of frequent occurrence of intense inflammation of the hilar and intrahepatic large bile ducts and also necroinflammation of the parenchyma and interface with portal inflammation in PSC recurrence remains speculative, the Roux loop reconstruction and superimposed ascending biliary infection may have a role to play in the formation of intense inflammation of the hilar and intrahepatic large bile ducts or could be related to immunosuppression therapy in such patients [11].

### *7.3.4 Outcomes for PSC Recurrence at Kyoto University Hospital*

In a study series conducted at Kyoto University Hospital, 47 patients underwent primary LDLT for end-stage PSC during the period June 1990–May 2016. Nine patients with original disease of PSC received a second graft, including one cadaveric graft, due to graft failure caused by recurrent PSC, and two patients received a third graft (cadaveric) due to recurrent PSC. The clinical details of ten patients who received primary LT and retransplantation for recurrent PSC in our hospital are shown in Table 7.1. Two patients who received primary grafts at other hospitals and were referred to Kyoto University Hospital for retransplantation and one patient who received a secondary graft at another hospital are included in these ten patients. A part of the follow-up data on these patients were provided in our previous report [3].

**Table 7.1** Clinicopathological details of patients who underwent retransplantation for recurrent PSC

| Patient | Gender | Age at LT (year) | Number of grafts[a] | Donor | PSC recurrence after LT (year) | Time interval between PSC and LT (year) | Histologic overlap AIH on explanted liver | Outcome/histologyat last biopsy (year) | Follow-up after last LT |
|---|---|---|---|---|---|---|---|---|---|
| 1 | F | | Native | | – | 3.6 | – | 1st LT | |
| | | 5 | 1st | BRD | 3.3 | 0.7 | Yes | 2nd LT | |
| | | 9 | 2nd | BRD | 2.1 | 8.1 | – | 3rd LT | |
| | | 19 | 3rd (cadaveric) | NBRD | 4.4 | | | Mild fibrosis (4.4 year) | Alive 5.3 year |
| 2 | F | | Native | | – | 4.4 | – | 1st LT | |
| | | 19 | 1st | BRD | 2.1 | 3.6 | Yes | 2nd LT | |
| | | 25 | 2nd | BRD | 0.9 | | | Bridging fibrosis (1.4 year) | Alive 13 year |
| 3 | F | | Native | | – | 1.2 | – | 1st LT | |
| | | 20 | 1st | BRD | 1.9 (PSC) 8.4 (overlap AIH) | 7.7 | Yes | 2nd LT | |
| | | 30 | 2nd | NBRD | 2.2 | | | Bridging fibrosis (8 year) | Dead 8.3 year. (colon cancer) |
| 4 | F | | Native | | – | 9 | – | 1st LT | |
| | | 34 | 1st | BRD | 5.2 | 4.1 | Yes | 2nd LT | |
| | | 42 | 2nd | BRD | 1.1 (overlap AIH) | | | Bridging fibrosis (6.1 year) | Alive 8 year |
| 5 | M | | Native | | – | 20 | – | 1st LT | |
| | | 24 | 1st | BRD | 5.3 | 2.7 | – | 2nd LT | |
| | | 32 | 2nd | BRD | 4.9 | | | Precirrhosis (6.1 year) | Alive 8.4 year |
| 6 | M | | Native | | – | 3 | – | 1st LT | |
| | | 25 | 1st | BRD | 4 | 2 | – | 2nd LT | |
| | | 31 | 2nd | BRD | 5.9 | | | Mild fibrosis (8.5 year) | Alive 9.2 year |

(continued)

**Table 7.1** (continued)

| Patient | Gender | Age at LT (year) | Number of grafts[a] | Donor | PSC recurrence after LT (year) | Time interval between PSC and LT (year) | Histologic overlap AIH on explanted liver | Outcome/histologyat last biopsy (year) | Follow-up after last LT |
|---|---|---|---|---|---|---|---|---|---|
| 7 | F | | Native | | – | 8 | NA | (1st LT) | |
| | | 47 | 1st | NA | 1.7 | 1.3 | Yes | 2nd LT | |
| | | 50 | 2nd (cadaveric) | NBRD | | | | | Dead 2 wk. (sepsis, no PSC recurrence) |
| 8 | M | | Native | | – | 8 | NA | (1st LT) | |
| | | 21 | 1st | BRD | 3 (PSC), 5.6 year. (overlap AIH) | 4 | Yes | 2nd LT | |
| | | 28 | 2nd | BRD | 4.7 | 4.5 | – | 3rd LT | |
| | | 37 | 3rd (cadaveric) | NBRD | 0.9 (overlap AIH) | | | Mild fibrosis (0.9 year) | Dead 3.8 year (PSC recurrence) |
| 9 | F | | Native | | – | 12 | – | 1st LT | |
| | | 14 | 1st | BRD | 2.2 | 2.2 | NA | (2nd LT) | |
| | | 19 | 2nd (cadaveric) | NBRD | 3.1 | | | Mild fibrosis (3.1 year) | Alive 9 year |
| 10 | F | | Native | | – | 7 | – | 1st LT | |
| | | 30 | 1st | BRD | 5.5 | 6 | – | 2nd LT | |
| | | 42 | 2nd | NBRD | 4.5 | | | Bridging fibrosis (4.5 year) | Alive 5.7 year |

*AIH* autoimmune hepatitis, *BRD* blood-related donor, *NBRD* non-blood-related donor, *NA* not available, *LT* liver transplantation, *PSC* primary sclerosing cholangitis, LT at other hospitals

[a]grafts are from living donors if not otherwise specified

The mean time from PSC diagnosis to primary LDLT was 7.6 years (range, 1.2–20 years). In contrast, the mean time interval between the diagnosis of PSC recurrence and retransplantation was 3.4 years (range, 0.7–7.7 years). With subsequent grafts, the interval to recurrence was reduced (mean of 3.4 years in the first graft (n = 10), 3.2 years in the second graft (n = 10), and 2.8 years in the third graft (n = 2)); this finding was consistent with data of a previous report of an LT series using livers from donation after brain death donors [23]. In this series, the median time to recurrence was 4.75 years (n = 50) in the first graft, 4.87 years (n = 8) in the second graft, 2.85 years (n = 2) in the third graft, and 0.56 years (n = 1) in the fourth graft [23].

Active hepatitis changes in addition to pure PSC were found in six explanted livers (first graft) at the time of retransplantation in the cases listed in Table 7.1. Before retransplantation, the finding of interface hepatitis was detected on allograft biopsy in only two of these six cases (Fig. 7.3a, b). Interestingly, these two patients

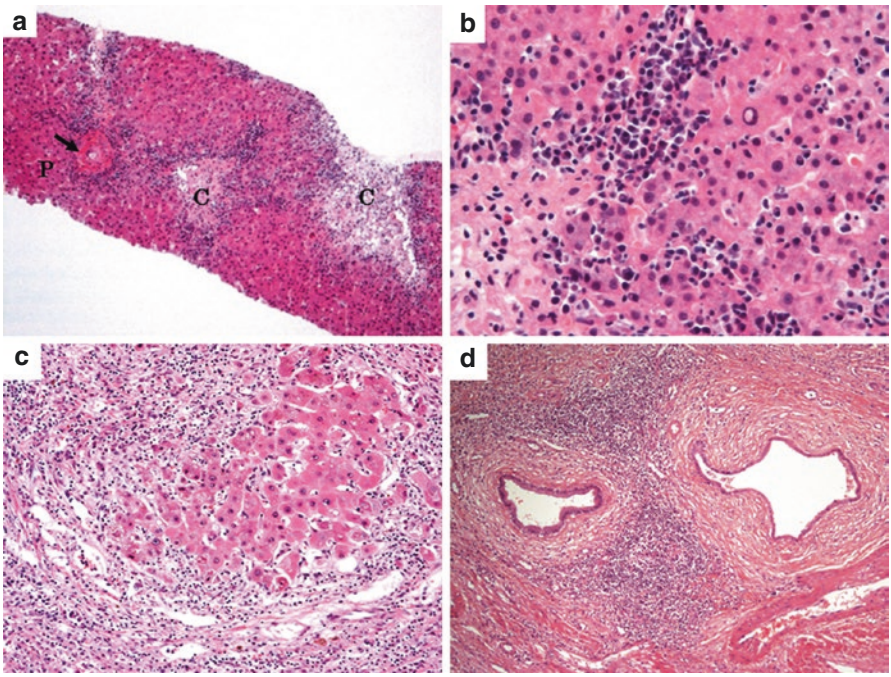


**Fig. 7.3** Recurrent primary sclerosing cholangitis (PSC) with overlap of interface hepatitis. **a**: Biopsy obtained from liver of recurrent PSC with overlap interface hepatitis after living donor liver transplantation, showing the dense portal (P) and centrilobular (C) lymphoplasmacytic infiltrate with interface activity. Intrahepatic small bile ducts are missing (arrow). Note the uninvolved adjacent interlobular hepatic artery. **b:** Higher magnification of (**a**). Prominent plasma cell infiltration. **c**: Explanted liver allograft showing recurrent PSC with overlap interface hepatitis. Marked lymphoplasmacytic infiltration with interface hepatitis is evident in biliary-type cirrhosis. **d**: A septal bile duct of (**c**) showing periductal fibrosis with lymphocytic infiltration, compatible with recurrence of PSC

initially had pure PSC recurrence after LDLT and subsequently developed histologically AIH-like features after the diagnosis of PSC recurrence. Two of ten patients for the second graft and one of two patients for the third graft had findings of PSC/AIH overlap on biopsy. Two patients showing PSC/AIH overlap at second or third grafts had evidence of PSC/AIH overlap at the previous explanted grafts (Fig. 7.3c, d).

The overlap of AIH with PSC is more commonly seen in children than in adults [4–7]. In our patients, seven out of ten were under age 30 years at the time of the first LT. The female predominance and the rather younger age of our cases may be one of the causes of frequent overlap of active hepatitis in recurrent PSC. Only one patient showed manifestation of AIH and then developed bile duct abnormalities diagnostic of PSC on cholangiography over a 7-year period in the native liver. The original disease of the remaining nine patients was purely PSC, with no histologic or serologic evidence of AIH, suggesting that these patients were newly experiencing AIH/PSC overlap after LDLT [3].

### *7.3.5 Treatment for Recurrent PSC*

The primary immunosuppressive regimen for recurrent PSC patients has been a combination of tacrolimus and prednisolone in addition to ursodeoxycholic acid (UDCA). Triple immunotherapy (i.e., calcineurin inhibitor (cyclosporine or tacrolimus), azathioprine, and corticosteroids) may be effective [22, 23]. However, despite experiencing biochemical improvement with steroid therapy, most of the patients eventually developed liver cirrhosis followed by retransplantation in the Kyoto series [3].

In conclusion, in patients with recurrent PSC post-LDLT, (1) the post-LDLT course is more rapid than that before LDLT, (2) overlap of active hepatitis (AIH) is relatively common in comparison with native PSC, and (3) hilar xanthogranulomatous and lymphoplasmacytic inflammation are prominent in comparison with native PSC. Frequent overlap of active hepatitis and frequent occurrence of xanthogranulomatous cholangitis of the hilar bile ducts and intrahepatic large bile ducts may explain why some patients have a more aggressive course of disease with recurrences in sequential grafts.

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# Chapter 8
# Recent Advances in Pathology and Pathogenesis of IgG4-Related Sclerosing Cholangitis and Its Related Diseases

**Yasuni Nakanuma**

**Abstract** As a hepatobiliary manifestation of IgG4-related diseases, IgG4-sclerosing cholangitis (IgG4-SC) is a representative one and is associated with many lymphoplasmacytic infiltration and fibrosis, particularly IgG4-positive plasma cells. Among hepatobiliary pseudotumors, a majority of lymphoplasmacytic type is included in IgG4-SC with exaggerated inflammatory response. IgG4-associated AIH is characterized by extensive parenchymal necroinflammation in addition to portal and periportal hepatitis with many IgG4-positive plasma cells. A majority of pathological changes of IgG4-hepatopathy may be secondary to IgG4-SC and/or type I autoimmune pancreatitis (AIP). Th2 predominance, specific response with dominant IgG4(+)B-cell receptor clones, and Treg activation may be important immunologic processes associated with IgG4-SC. In addition, the findings that IgG4-SC and type I AIP share many histopathological features and occur in almost the same patients raise the possibility that the same or similar anatomical components, namely, the acini of the peribiliary glands and the exocrine pancreas, are the primary target of these diseases. While their histopathologies are easily recognizable, these diseases may clinically mimic various inflammatory and neoplastic hepatobiliary diseases, particularly cholangiocarcinoma (CCA). CCA cells may function as nonprofessional APCs and perform immunosuppressive functions similar to Treg cells via IL-10 production and may induce the differentiation of the IgG4-positive plasma cells (IgG4 tissue reactions) in CCA. Sophisticated approaches to differentiate IgG4-SC from primary sclerosing cholangitis are another challenging issue in the future.

**Keywords** IgG4-related disease • Sclerosing cholangitis • Autoimmune pancreatitis • Autoimmune hepatitis • Cholangiocarcinoma

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## Abbreviations

| | |
|---|---|
| AIH | Autoimmune hepatitis |
| AIP | Autoimmune pancreatitis |
| ANAs | Antinuclear antibodies |
| APC | Antigen-presenting cell |
| BCR | B cell receptor |
| CA-II | Carbonic anhydrase-II |
| CCA | Cholangiocarcinoma |
| IgG4-RD | IgG4-related disease |
| IgG4-SC | IgG4-related sclerosing cholangitis |
| LF | Lactoferrin |
| PSC | Primary sclerosing cholangitis |
| SC | Sclerosing cholangitis |
| Treg | Regulatory T cell |

## 8.1 Introductory Remark

Since Hamano et al. [1] first linked autoimmune pancreatitis (AIP), now known as type I AIP, to elevated serum levels of IgG4, diseases pathologically and clinically similar to type I AIP have been described in almost every organ of the body [2–4]. These diseases mainly affect middle-aged and elderly males, while children and youths are not affected, and are characterized by elevated serum IgG4 values (usually ≥135 mg/dl), marked IgG4-positive plasmacytic cell infiltration in the affected organs, and marked efficacy of steroid therapy. These diseases are collectively referred to as IgG4-related disease (IgG4-RD) [2–4]. Pathologically, marked lymphoplasmacytic infiltration, including a large number of IgG4-positive plasma cells, and storiform fibrosis usually resulting in a tumorous lesion and obliterative phlebitis are common characteristic findings. IgG4-RD in a given organ is occasionally associated with another IgG4-related disease in other organ, either synchronously or metachronously [2, 3].

In the hepatobiliary system, IgG4-sclerosing cholangitis (IgG4-SC) is considered an IgG4-RD and mainly involves the extrahepatic bile ducts and occasionally the hilar and intrahepatic large bile ducts [2, 5–8]. In addition, IgG4-related inflammatory pseudotumor, IgG4-hepatopathy, and IgG4-related autoimmune hepatitis have also been described as IgG4-RDs. Of note, type 1 AIP and IgG4-SC frequently develop in the same patient.

We herein report the recent advances in IgG4-SC and its related hepatobiliary diseases, with an emphasis on their pathology and pathogenesis. We will also discuss whether or not IgG4-SC is related to biliary malignancy and touch on the significance of an IgG4 reaction in cholangiocarcinoma (CCA).

## 8.2 IgG4-SC and Its Related Diseases

### *8.2.1 IgG4-SC*

IgG4-SC affects large bile ducts, including the hilar and intrahepatic large bile ducts and extrahepatic bile ducts, and induces biliary stenosis with cholangitis and obstructive jaundice [2, 5]. IgG4-SC is considered a "benign" disease with a low risk of liver failure and responds well to steroid therapy.

#### 8.2.1.1 Pathology

Grossly, the affected bile ducts usually show diffuse and circumferential wall thickening, although uneven involvement of the ductal wall is also occasionally encountered [2, 5]. The lumen of the affected bile ducts is stenotic, and the mucosal surface is relatively smooth with no ulceration. The extrahepatic bile duct, particularly its intrapancreatic portions, is frequently affected, and the hilar or intrahepatic large bile ducts are also occasionally affected as well.

Histologically, IgG4-SC induces transmural fibroinflammation, with even distribution from the mucosal surface to the subserosa, although the lining surface epithelium remains relatively intact despite the severe fibroinflammation of the duct wall (Fig. 8.1). In addition to the ductal wall, the peribiliary glands are also usually severely affected, and glandular acini-oriented necroinflammation is frequently clearly recognizable (Fig. 8.2, 3). The dense infiltration of inflammatory cells can be seen, comprising mainly lymphocytes and plasma cells (Fig. 8.1b). Eosinophilic infiltration is also observed in most cases, with notable eosinophilic infiltration in some cases, whereas neutrophils are rare. Sclerosing inflammation with lymphoplasmacytic infiltration also occasionally extends along nerve fibers and into the fibroadipose connective tissue around the bile duct (Fig. 8.1a). This characteristic pattern of fibrosis is called "storiform fibrosis," in which collagen fibers are arranged in an irregular whorled pattern (Fig. 8.1c). The fibroinflammation is known to involve veins, leading to partial or complete obliteration (obliterative phlebitis), which is characteristic of IgG4-RDs in general and easily identifiable by EVG staining (Fig. 8.1d).

Immunostaining for IgG4 reveals the massive infiltration of IgG4-positive plasma cells (Fig. 8.1e), although their density and distribution vary among the affected ducts and periductal tissues. The cutoff values for concentrations of IgG4+ plasma cells proposed for diagnosing IgG4-SC are 50 cells/hpf for surgical specimens and 10 cells/hpf for biopsy samples. In addition, the ratio of IgG4-positive to total IgG-positive plasma cells exceed 40% [2]. In differential diagnosis of IgG4-SC from other hepatobiliary diseases, this semiquantitative assessment is helpful but not absolute.

### 8.2.1.2 Pathogenesis of IgG4-SC

The pathogeneses of IgG4-RDs are multifactorial and may be similar across multiple organs [2, 3]. In addition, environmental factors and genetic backgrounds are also typically involved in the development of IgG4-RDs.

#### Immunopathogenesis

Innate and acquired immunity, Th2-dominant immune reactions, and regulatory T (Treg) and B cell activation may all be involved to some degree in the development of IgG4-RDs [3, 9, 10].

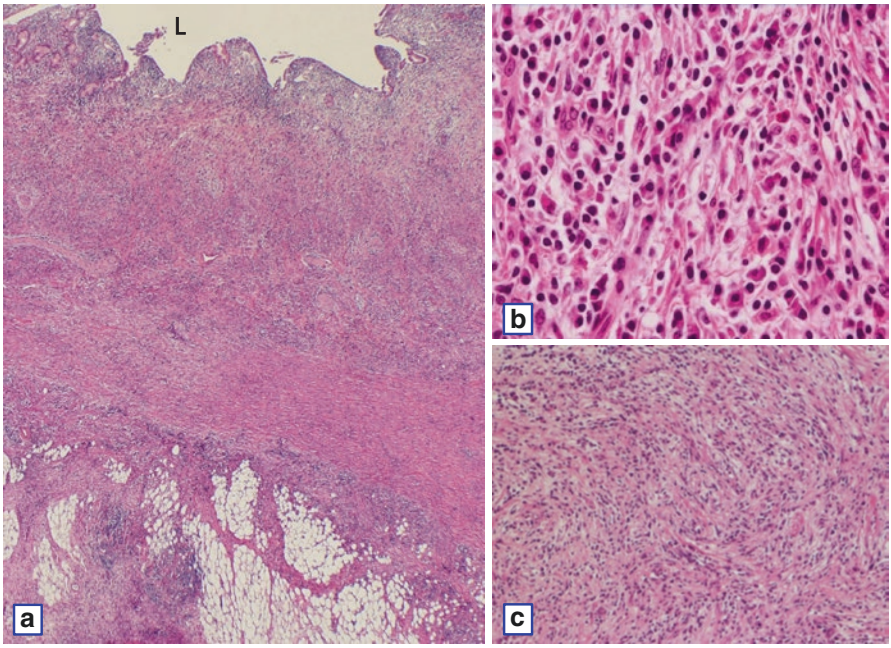
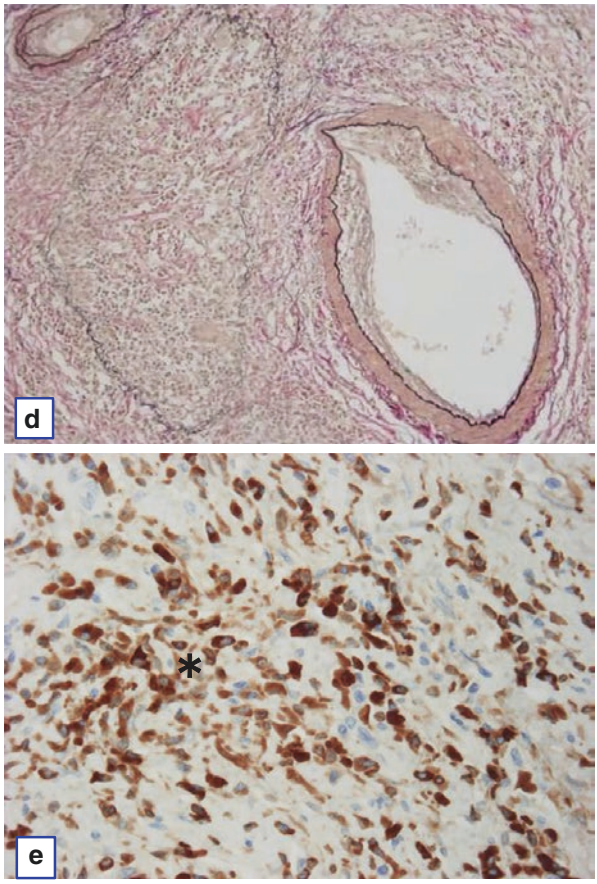



**Fig. 8.1** Histopathologies of IgG4-related sclerosing cholangitis. **a**: The bile duct wall is thickened and fibrotic, and the lymphoplasmacytic infiltration is extensive. The fibroinflammatory changes extend into the preductal fatty tissue. L, bile duct lumen. H&E, x80 (original magnification). **b**: The lymphoplasmacytic infiltration with eosinophils. H&E, x300 (original magnification). **c**: Storiform fibrosis with the lymphoplasmacytic infiltration is evident in the fibrotic duct wall. H&E, x150 (original magnification). **d**: The vein (*) is obliterated by the fibroinflammatory changes (obliterative phlebitis). EVG staining, x120 (original magnification). **e**: Many IgG4-positive plasma cells have infiltrated the tissue. Immunostaining of IgG4, x300 (original magnification)

**Fig. 8.1** (continued)




*Humoral Immune Responses*

Antinuclear antibodies (ANAs) and other autoantibodies are not infrequently detected, and levels of γ-globulin and IgG, particularly IgG4, are elevated, suggesting an abnormality in the humoral immune system [3]. Although details regarding the role of IgG4 in IgG4-RDs remain unclear, an elevated serum level of IgG4 is associated with the disease activity, and IgG4 production is upregulated by IL-10 from Treg cells and by B cell-activating factors. However, while IgG4 is clearly a key immunoglobulin involved in IgG4-RDs, no IgG4-type autoantibodies have been detected in patients with IgG4-RDs, including IgG4-SC.

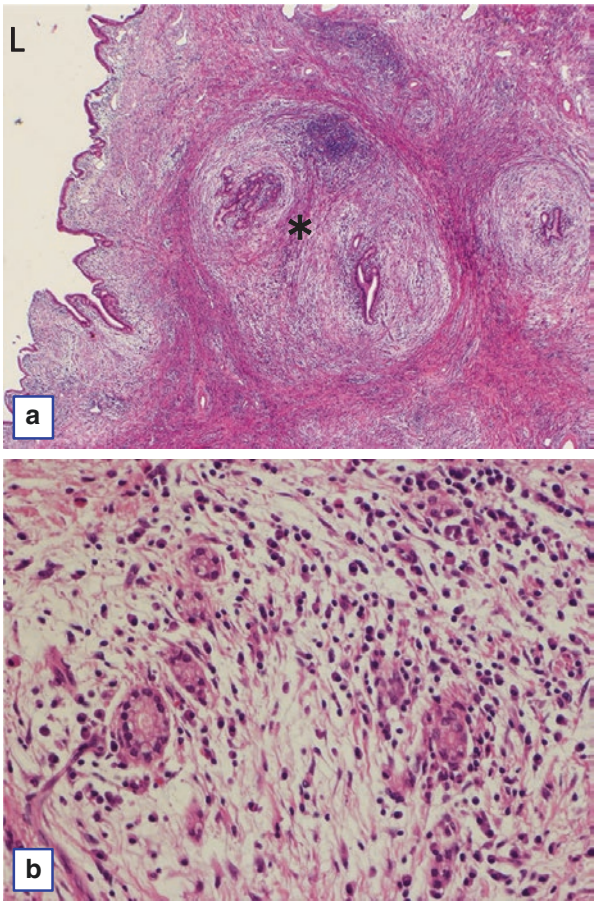



**Fig. 8.2** Involvement of peribiliary glands in IgG4-related sclerosing cholangitis. **a**: The fibroinflammatory changes are oriented to the acini of the peribiliary glands (*). H&E, x80 (original magnification). **b**: The affected acini of the peribiliary glands show some destruction. H&E, x250 (original magnification)

*T Cell Immune Responses*

T-helper (Th)2 lymphocytes and regulatory T cells (Tregs) are upregulated in IgG4-RDs [10]. Th2 cytokines such as IL-4, IL-5, and IL-13 are significantly overexpressed, and Th2 cytokines may be involved in the disease progression, especially the maturation and proliferation of B cells and plasmacytes. The Th2-dominant immune reactions appear to be a reasonable explanation for the serum eosinophilia and elevated IgE concentrations. Large numbers of FOXP3+/CD4+/CD25+ Tregs have been observed in the bile duct tissue of patients with IgG4-SC, along with the overexpression of two regulatory cytokines (IL-10 and TGF-β). IL-10 and IL-4 are suspected to participate in an IgG4 class switch in B cells, followed by the increased production of IgG4. TGF-β may be responsible for fibrosis of the affected bile ducts.

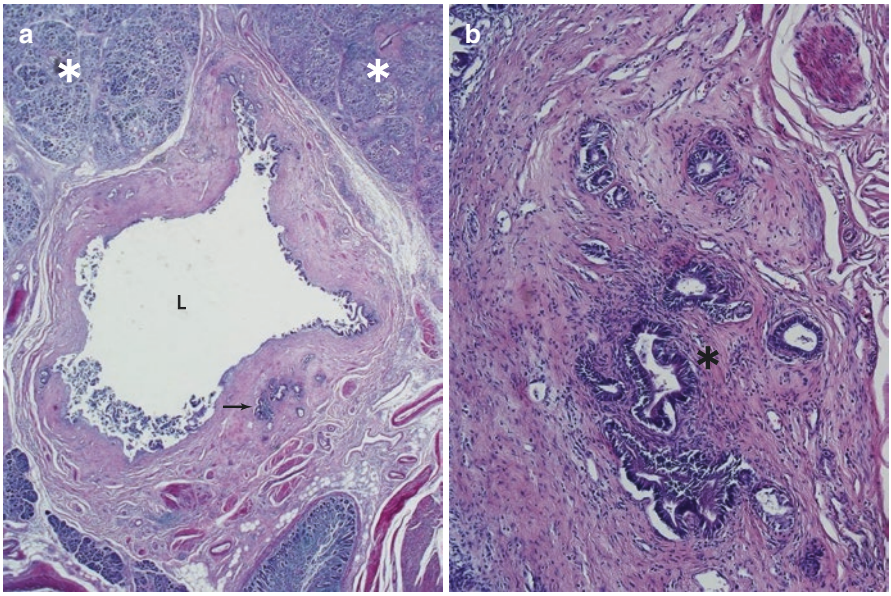



**Fig. 8.3** The distal bile duct of type I autoimmune pancreatitis. **a**: Some parts (→) of the apparently normal bile duct show inflammatory changes. The surrounding pancreas (*) shows extensive inflammatory changes with acinar destruction. H&E, x80 (original magnification). **b**: Higher magnification of A. Peribiliary glands (*) show lymphoplasmacytic infiltration. H&E, x150 (original magnification)

*B Cell Immune Responses*

The findings from recent studies have suggested that specific B cell responses may play an important role in the pathogenesis of IgG4-SC [9]. In addition to steroid and immunomodulators, B cell depletion by rituximab, a monoclonal CD20 antibody, is reported to be a useful and promising therapeutic strategy against IgG4-RDs. Interestingly, rituximab reduces only the IgG4 subclass, with no effects on the subclasses of IgG1, IgG2, or IgG3.

Recently, dominant IgG4+ B cell receptor (BCR) clones were identified using next-generation sequencing in the peripheral blood of patients with active IgG4-SC but not in healthy or disease controls [9]. Of note, the same clones were encountered in inflamed tissues, such as the duodenal papilla, and comprised the same dominant IgG4+ clones as the paired peripheral blood samples. This expansion of IgG4+ BCR clones into the blood and tissue of patients with active IgG4-SC can be abrogated with corticosteroid treatment [9]. Taken together, these previous findings suggest that clonally expanded, class-switched IgG4-positive B cells and plasma cells may be causal immunopathological features of IgG4-SC. Furthermore, the detection or identification of these BCR clones may prove useful for differentiating

IgG4-SC from other chronic biliary diseases in which these BCR clone are not detected, such as primary sclerosing cholangitis (PSC) and CCA.

## Target Tissues or Antigens

While IgG4-RDs have been identified in a number of organs throughout the body, these diseases tend to be accompanied by clustering of affected organs. IgG4-RD patients can be classified into several groups based on the location of the affected organs, namely, head and neck, thoracic, hepatic and pancreatobiliary, and retroperitoneal groups [4]. Group-specific features have been reported; for example, the proportion of female patients and the serum IgG4 concentrations are both significantly higher in the head and neck group than in other groups. These features may reflect different manifestations of a single disease entity or suggest different underlying etiologic factors with similar clinicopathological features or unique target antigens among groups.

### *Similarities in the Affected Anatomical Components Between IgG4-SC and Type I AIP*

Among IgG4-RDs, IgG4-SC and AIP are more frequently observed in the same individual than with other IgG4-RDs. IgG4-SC and type I AIP present characteristic histopathological features, such as dense lymphoplasmacytic infiltration and extensive fibrosis with a storiform pattern, suggesting that the same anatomical components or antigens in the hepatobiliary and pancreatic systems are targeted and attacked by the same mechanisms between the two diseases [11] (Fig. 8.4).

Indeed, in type I AIP, characteristic necroinflammation of the exocrine acini of the pancreas is consistently observed, while in IgG4-SC, necroinflammation is generally observed with the acini of the peribiliary glands, which are physiologically distributed around the large bile ducts selectively affected by IgG4-SC. Of note, small populations of pancreatic exocrine acini are intermingled with these peribiliary glands, and from a physiological aspect, the biliary tract could actually be regarded as an incomplete pancreas [11], raising the possibility that some antigens located in the pancreatic exocrine acini and the peribiliary gland acini might be a common target of immunological attack in type I AIP and IgG4-SC (Fig. 8.4). This might explain pathoanatomically why the extrahepatic and intrahepatic large bile ducts and the exocrine pancreas are more severely affected, simultaneously, than other organs by these diseases.

As mentioned above, the peribiliary glands are affected in almost every case of IgG4-SC with type I AIP (Fig. 8.2a, b). However, in some cases of type I AIP without biliary involvement by gross and radiological examinations, the extrahepatic bile ducts have shown mild inflammatory change with lymphoplasmacytic infiltration in the peribiliary glands, while other bile duct and periductal components seem spared from necroinflammation (Fig. 8.3a, b). Although IgG4-SC typically induces

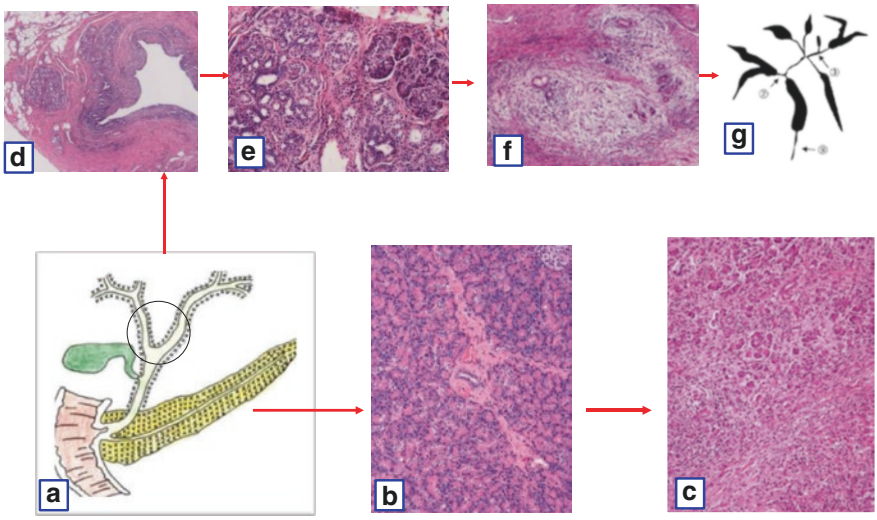



**Fig. 8.4** A schematic illustration depicting how IgG4-related sclerosing cholangitis (IgG4-SC) and type I autoimmune pancreatitis (AIP) develop in the same patient. **a**: A schematic illustration of the pancreas and biliary tree and duodenum. The peribiliary glands are located around the biliary tree (dots along the biliary tree). **b** and **c**: The exocrine acini of the pancreas (**b**) are targeted by type I AIP and show extensive destruction replaced by inflammation and fibrosis (**c**). **b** and **c**, H&E staining. **d, e,** and **f**: The peribiliary glands around the bile duct (*) (**d**) comprise serous and mucinous acini and occasionally pancreatic exocrine acini (**e**). The peribiliary glands are targeted by IgG4-SC and show inflammation and destruction (**f**). **g**: A schematic cholangiogram of the biliary tree of IgG4-SC shows stenosis of the distal portion and perihilar portions due to fibroinflammation induced by IgG4SC

transmural fibroinflammation, such cases as these suggest that the peribiliary glands are preferentially affected and may be the site of the initial pathologic lesion before progression to fully developed IgG4-SC (Fig. 8.3).

*Target Antigens*

In a considerable number of type I AIP cases, several autoantibodies have been reported. Some are non-organ specific, such as antinuclear antibodies (ANAs) and rheumatoid factor, while organ-specific ones, such as autoantibodies against lactoferrin (LF), carbonic anhydrase (CA)-II, CA-IV, and pancreatic secretory trypsin inhibitor, have also been observed [3]. Interestingly, the organ-specific antigens are all distributed in the exocrine acini and the duct epithelia of the pancreas. It therefore seems likely that these antigens located in the pancreas as well as in the peribiliary glands are targets in IgG4-SC as well as type I AIH. Indeed, the findings following immunization with CA-II- or LF-induced systemic lesions such as pancreatitis and cholangitis in mouse models of disease similar to human IgG4-RDs have supported this hypothesis.

## *8.2.2 Other Hepatobiliary Lesions Related to IgG4-SC*

### 8.2.2.1 Hepatobiliary Inflammatory Pseudotumor

Three types of inflammatory pseudotumors have been observed in the hepatobiliary system; myofibroblastic, lymphoplasmacytic, and fibrohistiocytic types, although these lesions are occasionally mixed. The lymphoplasmacytic type was recently reported to be related to IgG4-RD. This type pathologically presents with obliterative phlebitis and storiform fibrosis in addition to abundant lymphoplasmacytic infiltration. Interestingly, large proportions of IgG4-positive plasma cells have been found among IgG-positive plasma cells. Hepatic inflammatory pseudotumor itself is known to be associated with chronic cholangitis [12], and IgG4-SC presents as a tumorous lesion, and some cases may manifest as mass lesions typically involving perihilar ducts and intrahepatic large bile ducts. Based on both the clinical and pathological findings, such cases should be referred to as IgG4-related inflammatory lymphoplasmacytic pseudotumors. As such, a majority of, but not all, cases of lymphoplasmacytic-type hepatobiliary inflammatory pseudotumor may be regarded as a manifestation of IgG4-SC with an exaggerated inflammatory mass [6].

### 8.2.2.2 IgG4-AIH

Umemura et al. proposed referring to AIH characterized by chronic active hepatitis, high serum IgG4 levels, and abundant IgG4-positive plasma cell infiltration in the liver as "IgG4-associated AIH," stating that this type of AIH belongs to a spectrum of systemic IgG4-RDs [7]. Interestingly, IgG4-associated AIH is frequently complicated by well-known IgG4-RDs; however, only three cases (a 54-year-old female, a 42-year-old male, and a 73-year-old male) have been reported thus far [13].

These three cases described above all showed histological features similar to those of so-called chronic hepatitis with very-high-grade necroinflammation targeting hepatocytes, including marked portal inflammation comprised mainly of lymphoplasmacytes, marked interface hepatitis, and zonal-to-bridging necrosis. Plasma cell infiltration was prominent in portal tracts, and the proportion of IgG4-positive plasma cells among IgG-positive plasma cells exceeded 40% at the time of diagnosis. The parenchyma contained multiple foci of necrosis, and rosette formation was frequently observed. Substantial multinuclear giant cell transformation of hepatocytes was also evident in two cases. Interestingly, no biliary damage was noted. Serologically, the serum IgG4 level exceeded 135 mg/dL, and ANA was positive in all cases. All three IgG4-associated AIH cases responded well to steroid therapy, just as is commonly seen in classical AIH. Discontinuation or reduction of steroid treatment resulted in recurrence of IgG4-associated AIH in two cases. However, among the IgG4-associated AIH cases reported thus far, none showed any obliterative phlebitis, storiform fibrosis, or fibroinflammatory tumorous lesions in the liver.

#### 8.2.2.3 IgG4-Hepatopathy

Variable hepatic dysfunctions are observed frequently in cases of type 1 AIP with or without IgG4-SC. In addition, variable histopathologic changes, including infiltration of IgG4-positive plasma cells, have been also encountered in needle liver biopsies from or in the peripheral parts of surgically resected livers with either or both type I AIP and IgG4-SC. Umemura et al. recently proposed collectively calling these lesions "IgG4-hepatopathy" [8], and they classified the pathological changes of "IgG4-hepatopathy" into the following five patterns: (i) portal inflammation, (ii) large bile duct damage, (iii) portal sclerosis, (iv) lobular hepatitis, and (v) cholestasis.

Interestingly, multiple histological patterns can coexist in the same case. The portal inflammation pattern is characterized by infiltration of mononuclear cells containing plasma cells into the small portal tracts, occasionally associated with interface hepatitis. Portal inflammation may be a part of IgG4-SC. The large bile duct damage pattern is characterized by ductular proliferation, neutrophil infiltration, and edematous change in the portal areas. Furthermore, portal tract fibrosis may be secondarily associated with SC involving the large or extrahepatic bile ducts. The lobular hepatitis pattern may resemble mild viral hepatitis, for example, as a parenchymal inflammation pattern in conjunction with hepatocellular focal necrosis. Finally, the cholestatic pattern is a canalicular cholestasis found predominantly in the centrilobular area and is consistent with extensive stenosis and obstruction of the affected extrahepatic bile ducts, including the distal bile ducts. While the histological lesions of IgG4-hepatopaty are heterogeneous, some of these lesions may simply be secondary to the biliary involvement of either or both IgG4-SC or type I AIP, and others may be hepatic lesions inherent to systemic IgG4-RDs.

#### 8.2.2.4 Other Lesions

The gallbladder is commonly involved (IgG4-related sclerosing cholecystitis) in patients with either or both IgG4-SC or type I AIP, and IgG4-related sclerosing cholecystitis shows similar morphologies to IgG4-SC [2, 14]. The endoscopic features of the major and minor papillae were abnormal in 44% and 38% of the patients with type I AIP, and abundant infiltration of IgG4-positive plasma cells was more frequent in the patients with abnormal major papilla than in those with normal major papilla [2, 15].

### *8.2.3 Differential Diagnosis of IgG4-SC from Other Hepatobiliary Diseases*

IgG4-SC and other hepatobiliary diseases mimic inherent hepatobiliary diseases such as primary sclerosing cholangitis (PSC), CCA, and other forms of SC with lymphoplasmacytic infiltrates [2].

#### 8.2.3.1 PSC

PSC and IgG4-SC target large bile ducts, such as the hepatic hilar bile duct and extrahepatic bile ducts. Although IgG4-SC is characterized by the infiltration of numerous IgG4-positive plasma cells in the bile duct walls, this IgG4 reaction is also found in a small percentage of cases of PSC and hepatolithiasis, another form of secondary sclerosing cholangitis. Clinically, differentiating IgG4-SC cases without other organ involvement, including type I AIP, from PSC may be challenging. Unlike IgG4-SC, PSC generally shows more mucosa-oriented tissue damage with frequent ulceration. Obliterative phlebitis and storiform fibrosis support the diagnosis of IgG4-SC, while the neutrophilic infiltration and obliteration of the affected bile ducts suggest PSC.

#### 8.2.3.2 CCA

IgG4-SC clinicopathologically mimics hilar and extrahepatic CCA. Differentiating hilar CCA from IgG4-SC cases may therefore be particularly difficult in cases without type I AIP. More than 40% IgG4-positive plasma cells and >10 cells/hpf in biopsy samples are comprehensive histological diagnostic criteria for IgG4-RDs and may support the diagnosis of IgG4-SC but only if malignant neoplasms can be excluded [2].

Of note, mass formation and marked IgG4-positive cell infiltration are occasionally found in CCA, as is the variable infiltration of IgG4-positive plasma cells and an inflammatory reaction characterized by large numbers of IgG4-positive plasma cells within or around the tumors. Resheq et al. also recently showed that one third of patients with hilar CCA were IgG4-positive (≥20 IgG4-positive plasma cells/hpf) [16], and they suggested that IgG4-positive plasma cells are of limited utility in distinguishing hilar CCA from IgG4-SC, even when combined with clinical parameters, and may actually be misleading in situations where malignancy has escaped notice [16].

## 8.3 IgG4-SC and CCA

Whether or not the presence of IgG4-SC increases the long-term risk of CCA remains unclear [17–19]. However, several case reports have been published regarding IgG4-SC associated with CCA or its precursor lesion biliary intraepithelial neoplasia (BilIN) [16]. In addition, biliary cancers arising from IgG4-RD have been reported [19], although no study has yet conclusively established a cause-and-effect relationship between IgG4-RD and CCA.

### *8.3.1 IgG4-RD and Cancer*

Recent reports have demonstrated that patients with type I AIP are occasionally complicated with various types of cancer, including pancreatic cancer and CCA. Shiokawa et al. reported that, among 108 type I AIP patients, 18 cancers in

various organs were found in 15 patients (13.9%) during a median follow-up period of 3.3 years [19]. Prior to initiating corticosteroid therapy for type I AIP, numerous IgG4-positive plasma cells were observed in the cancer stroma, and no patients had type I AIP relapse after successful cancer treatment. Given the observed high risk for developing cancer during the first year after a type I AIP diagnosis and the absence of type I AIP relapse after successful treatment of coexisting cancers, the authors concluded that type I AIP may develop as a paraneoplastic syndrome in some type I AIP patients. However, Hirano et al. [18] surveyed 113 patients with IgG4-related disease in whom malignancy was not diagnosed at the time of IgG4-RD onset and found that the incidence of the observed malignancies was not significant. Prospective and megastudies will be required to clarify this issue conclusively.

### *8.3.2 CCA and IgG4-SC*

A few cases of IgG4-SC associated with CCA or its precursor lesion have been reported [17, 20]. BilIN has been defined as a precursor of CCA and classified into three subtypes: BilI1, BilIN2, and BilIN3. BilIN3, an in situ carcinoma, and BilIN1-2 have been found in patients with IgG4-SC, suggesting that these cases were at risk for progression of invasive CCA. BilIN lesions have also been found to express a mutated form of the p53 tumor suppressor protein, suggesting that CCA may be associated with IgG4-SC as a precursor of malignancy [20].

### *8.3.3 CCA and IgG4 Tissue Reaction*

#### 8.3.3.1 Infiltration of the IgG4+ Plasma Cells in CCA

Several cases of carcinomas accompanied by IgG4-positive plasma cells within and around carcinoma tissue (IgG4 tissue reaction) and increased serum IgG4 levels have been reported. With CCA, such IgG4 reactions are known to occur to various degrees in CCAs located along the biliary tree, except for intrahepatic CCA. Furthermore, about one third of these CCA cases exhibited >10 IgG4-positive plasma cells/hpf, meeting the clinical and pathologic diagnostic criteria of IgG4-SC (Fig. 8.5a, b) [16, 21, 22].

#### 8.3.3.2 IgG4 Tissue Reaction Reflects the Evasion of Immunosurveillance by Treg Cells

During the carcinogenesis of pancreatic cancer, the number of Foxp3+ Treg cells increases, while that of CD8+ cytotoxic T cells (CTLs) decreases, suggesting that Treg cells are involved in the immune response against pancreatic cancers that evade tumor-associated immunosurveillance [23]. Treg cells inhibit anticancer immunity of the host by producing regulatory cytokines, such as IL-10 and TGF-β, and high Treg cell frequency is speculated to reflect a poor

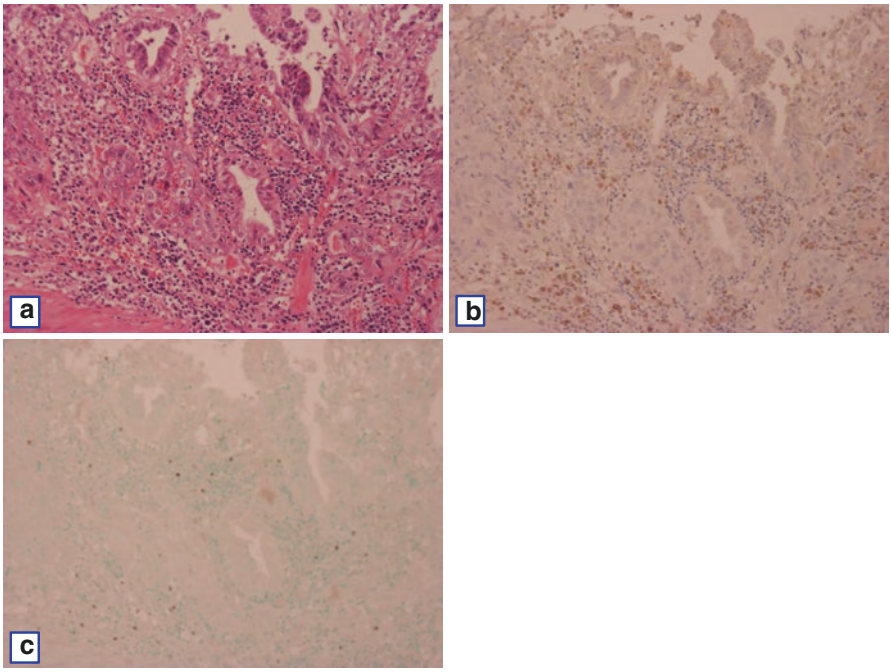



**Fig. 8.5** Cholangiocarcinoma of the extrahepatic bile duct and IgG4 tissue reaction. **a**: Tubular adenocarcinoma with extensive lymphoplasmacytic infiltration; H&E, x200 (original magnification). **b**: The IgG4-positive plasma cells show dense infiltration. Immunostaining of IgG4, x200 (original magnification). **c**: Foxp3+ regulatory T cells are present in the inflammatory infiltrate. Immunostaining of Foxp3, x200 (original magnification)

prognosis in pancreatic cancer patients. The regulatory cytokine IL-10 produced by Treg cells may induce IgG4-positive plasma cell differentiation or promote B cell switching to IgG4 in the presence of IL-4. Treg cells are speculated to perform a similar function in the pathogenesis of IgG4-SC, as well as in the IgG4 tissue reaction of carcinoma.

In an examination of extrahepatic CCA cases, Kimura et al. reported that their immunohistochemistry analysis showed IgG4+ cells to be positively correlated with Foxp3+/CD4+ Treg but negatively correlated with CD8+ CTLs, suggesting the evasion of immunosurveillance associated with CD8+ cytotoxic T cells via the regulatory function of Foxp3+/CD4 + Treg (Fig. 8.5c) [21]. CD8+ CTLs exert immune activities similar to intraepithelial lymphocytes against cancers and invade cancerous nests. Consequently, the patients with a large number of these CD8+ CTLs were associated with scant IgG4 reactions. In contrast, IgG4-rich cases have few CD8+ CTLs and a poorer prognosis than IgG4-poor cases. Taken together, these findings suggest that the IgG4 reaction has a positive and negative correlation with Treg cells and CTLs, respectively, signifying that immunosurveillance evasion is associated with CTLs through the Treg cell regulatory function with the IgG4 tissue reaction.

#### 8.3.3.3 Cholangiocarcinoma Cells as Nonprofessional Antigen-Presenting Cells (APCs)

Immunocompetent cells like dendritic cells and nonimmunocompetent cells such as carcinoma and normal epithelial cells express MHC class II and may present antigens. MHC class II-positive cells that do not express the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) induce IL-10-producing anergic T cells [22, 24]. Several studies have suggested that antigen presentation by MHC class II-positive epithelial cells that lack costimulation signals promotes anergic T cell generation [24]. Carcinoma cells expressing MHC class II but lacking costimulatory molecules are found in about a half of the cases of biliary tract cancer [24]. These biliary tract cancer cells may function as nonprofessional APCs by generating IL-10-producing regulatory T cells (anergic T cells). Furthermore, an IL-10-predominant cytokine milieu could induce the production of IgG4-positive cells [22].

Although Foxp3 is a master transcription factor for Treg cells, Foxp3 and IL-10 are expressed in several carcinoma tissues and cultured cancer cell lines, suggesting that cancer cells induce the generation of a Treg cell-like immunoregulatory milieu to evade immunosurveillance [23, 24]. The findings from our study showed that CCA cells themselves express Foxp3 and function in immunosuppression in a manner similar to Treg cells [21, 22].

## 8.4 Conclusion

IgG4-SC and its related hepatobiliary diseases are an emerging disease affecting the hepatobiliary system. IgG4-SC is associated with many lymphoplasmacytic infiltration and fibrosis, particularly IgG4-positive plasma cells. IgG4-associated AIH is characterized by extensive parenchymal necroinflammation in addition to the histologies of chronic hepatitis with many IgG4+ plasma cells. Th2 predominance, specific B cell response with dominant IgG4+ receptor clones, and Treg activation may be involved in the immunologic pathogenesis of IgG4-SC. In addition, the acini of the peribiliary glands and the exocrine pancreas seem to be the primary target of IgG4-SC and type 1 AIP in the same patients. IgG4-SC may clinically mimic various inflammatory and neoplastic hepatobiliary diseases. IgG4-SC and CCA share several immunopathologic processes, and CCA cells may function as nonprofessional APCs that may act as Foxp3-positive and IL-10-producing cells directly inducing the IgG4 tissue reactions. Clinical and laboratory differentiation of IgG4-SC from CCA and PSC is a challenging issue in the future.

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# Chapter 9
# Pathology and Imaging of Peribiliary Cysts: Recent Progress

**Kazuto Kozaka and Osamu Matsui**

**Abstract** Peribiliary cysts are thought to be retention cysts, which arise in the peribiliary gland in a developmental or acquired manner. The former includes fibrocystic diseases such as autosomal dominant polycystic kidney disease (ADPKD), polycystic liver, and biliary hamartomatosis and the latter cirrhotic livers, especially in alcoholic-related cirrhosis, portal vein thrombosis, and idiopathic portal hypertension. With recent advances in imaging modalities and techniques, the incidence of detected peribiliary cysts has been dramatically increasing. Peribiliary cysts are usually 1 mm or less in diameter and are benign; however, when they cluster or are sufficiently large to compress adjacent bile duct, bile juice congestion may be evoked, resulting in clinical symptoms such as fever and jaundice due to cholangitis, forming hepatolithiasis, or obstructive jaundice. Furthermore, larger peribiliary cysts are sometimes difficult to distinguish from cystic hepatic tumors such as intraductal papillary neoplasm of the bile duct and mucinous cystic neoplasm of the liver. In this chapter, we outline the background pathology of peribiliary cysts as well as their imaging features and differential diagnosis.

**Keywords** Peribiliary cysts • Ultrasonography • Computed tomography • Magnetic resonance imaging • Magnetic resonance cholangiopancreatography

## 9.1 Introduction

Peribiliary glands composed of branched tubuloalveolar seromucinous glands are found around the extrahepatic and intrahepatic large bile ducts of humans at all ages [1]. These glands communicate with bile duct lumens through their own conduits and are relatively dense in the hilar bile ducts, cystic duct, and periampullary region [1–5].

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Peribiliary cyst was first reported by Nakanuma et al. in 1984 [6]. It is considered to be a retention cyst of the peribiliary gland and frequently associated with polycystic liver and chronic liver diseases especially alcoholic liver cirrhosis [2, 7–9]. In liver cirrhosis they gradually increase in size and number with disease progression. Peribiliary cyst is usually asymptomatic, but may cause obstructive jaundice due to bile duct compression by larger ones. In addition, clusters of peribiliary cysts in the hilar portal tracts mimic bile duct dilatation on imaging, and therefore knowledge of this pathology and imaging findings is important in clinical practice.

## 9.2 Anatomy, Histopathology, and Functions of Peribiliary Glands

Bile duct is the conduit of bile excretion from the liver to the gastrointestinal tract. For smooth excretion of bile, serous and mucous fluids are secreted from bile duct epithelium and peribiliary glands. Peribiliary glands can be divided into intramural and extramural types. The former are scattered within the bile duct walls and are simple tubular mucous glands. The latter are located in the periductal connective tissue and are branched tubuloalveolar seromucous glands (Fig. 9.1). The extramural glands drain into the large bile duct lumina via their own conduits, while the intramural glands drain directly into the bile duct lumen. Pancreatic acini without Langerhans islets are infrequently seen in the peribiliary glands with positivity for pancreatic

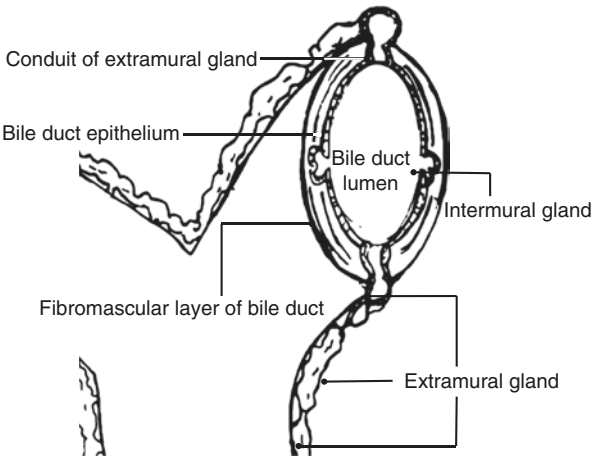


**Fig. 9.1** Scheme of normal large bile duct and peribiliary glands. Larger bile ducts, such as hilar bile ducts, cystic ducts, and periampullary region, have intramural (*arrowhead*) and extramural (*arrow*) peribiliary glands. The extramural glands drain into the large bile duct lumina via their own conduits, while the intramural glands drain directly into the bile duct lumen. Extramural glands branch out longitudinally along the bile ducts and anastomose with each other

exocrine enzymes. Intramural glands show hyperplasia in chronic cholangitis and secrete larger amounts of mucin. Extramural glands branch out longitudinally along the bile ducts and anastomose with each other [2, 3]. Peribiliary glands are densely observed in hilar bile ducts, cystic ducts, and periampullary region.

The functions of peribiliary glands are as follows: (1) secretion of several substances such as lactoferrin and lysozyme which damage bacteria, (2) formation of stem cell niches of the biliary tree which are capable of differentiating into hepatobiliary and pancreatic cells, (3) protection of the mucosal surface by secreting immunoglobulins, and (4) others. Stem/progenitor cells in the peribiliary glands are likely to be central to normal tissue turnover and injury repair and may play key roles in the pathophysiology of several biliary tract diseases [1, 3, 5, 10–12].

## 9.3 Pathophysiology and Clinical Significance of Peribiliary Cysts

Peribiliary cysts arise from the peribiliary glands of large intrahepatic and hilar bile ducts and therefore are commonly located in hilar and perihilar regions and are considered to be retention cysts of peribiliary glands. They are frequently multiple measuring a few millimeters to 1 cm in size, rarely up to 3 cm or more. In accordance with the distribution of peribiliary glands, peribiliary cysts are seen parallel to and surrounding the bile ducts (Fig. 9.2). The internal wall of the cysts is covered by a single layer of columnar or cuboidal epithelium (similar to bile duct epithelium) [1–3, 6–9].

At least two types of pathogenesis of peribiliary cyst are now considered [2, 13–15]. One is "acquired" as occasionally seen in cirrhotic livers, portal vein thrombosis, and idiopathic portal hypertension [2, 8, 9]. In cirrhotic livers, the frequency of peribiliary cysts increases in accordance with the progression of the disease.

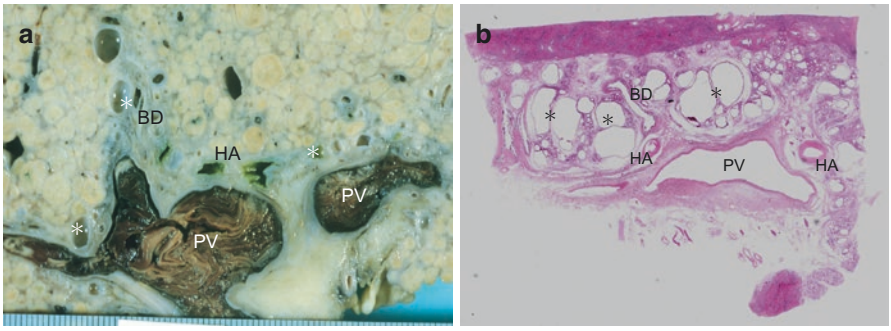


**Fig. 9.2** Pathology of peribiliary cysts. (**a**) Macroscopic picture of the liver with peribiliary cysts. Larger peribiliary cysts can be seen along the hilar portal tracts around portal tracts (*asterisks*). (**b**) Microscopically, variable-sized peribiliary cysts are shown around a larger bile duct (*BD*) (*asterisks*). *PV* portal vein, *HA* hepatic artery

Peribiliary cysts are more frequently seen in alcoholic liver fibrosis [8, 9] probably due to inflammation of pancreatic exocrine tissues in peribiliary glands [3, 5, 10, 11]. The other is "congenital or developmental" often associated with fibrocystic diseases such as autosomal dominant polycystic kidney disease (ADPKD), polycystic liver, and biliary hamartomatosis [2, 14, 15]. In the case of "congenital or developmental" etiology, the content of the cyst is usually serous and the wall is thin.

Peribiliary cysts are usually found incidentally by imaging, autopsy, and explanted liver in liver transplantation. However, they occasionally evoke intrahepatic bile duct dilatation due to the compression of bile ducts when they assume a large size and may cause jaundice, cholangitis, and hepatolithiasis [16].

Recently, some cases of cystic and papillary neoplasm involving peribiliary glands which are considered to be the biliary counterpart of branch-type intraductal papillary mucinous neoplasm of the pancreas have been reported [2, 3, 5, 17, 18]. The etiological relation between this entity and peribiliary cyst has not been verified, but a neoplastic lesion associated with cystic peribiliary glands should be always kept in mind in the differential diagnosis of cystic neoplasms in the hepatic hilum.

## 9.4 Imaging Findings of Peribiliary Cysts

For the evaluation of hepatobiliary diseases, ultrasound including contrast-enhanced ultrasound, Doppler and/or color Doppler ultrasound, dynamic CT, MRI including dynamic MRI and MR cholangiopancreatography (MRCP), percutaneous transhepatic cholangiography (PTC), endoscopic retrograde cholangiopancreatography (ERCP), and endoscopic ultrasound are useful. Gd-EOB-DTPA, a hepatobiliary contrast agent of MRI, can visualize bile ducts because around half of Gd-EOB-DTPA is taken up by hepatocytes and then is excreted into bile ducts around 10–20 min after intravenous injection. However, in spite of extremely high spatial resolution of multi-detector CT or endoscopic ultrasound, peribiliary glands cannot be visualized by imaging because of their microscopic size usually less than 1 mm. It is now considered that when a peribiliary cyst grows to more than a few millimeters (probably 3 mm or more), it can be depicted by imaging as a cystic lesion. In case of a cluster of microscopic peribiliary cysts, it may be demonstrated as a periductal solid mass lesion, because of its cavernous architecture like cavernous hemangioma of the liver.

Peribiliary cysts contain serous and/or mucinous fluid and therefore commonly show the imaging features of water collection in various imaging modalities. The majority are 2–3 mm in size with a thin cyst wall, but can be up to 30 mm and more. On imaging, peribiliary cysts are usually located surrounding larger bile ducts, namely, distal portion of right/left hepatic ducts and to their first- and second-order branches. They are rarely seen surrounding extrahepatic bile ducts.

On ultrasound they are depicted as round or tubular anechoic (cystic) lesions situated along the larger portal tracts often with a cluster of similar-sized lesions

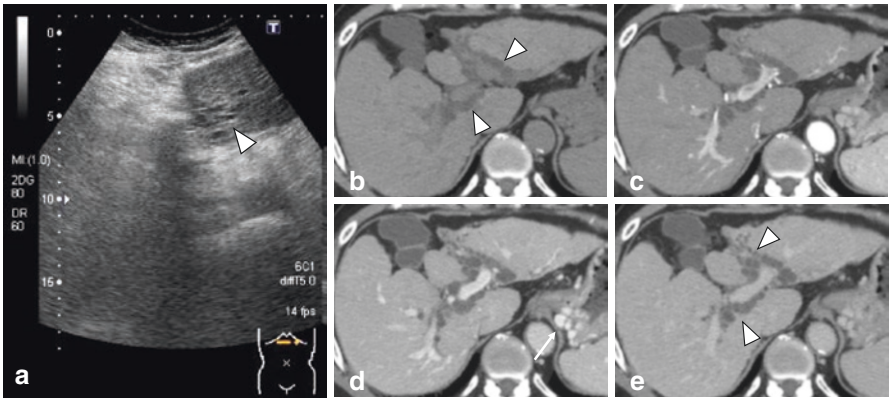


**Fig. 9.3** Ultrasonography and CT findings of peribiliary cysts. (**a**) B mode of ultrasonography shows congregated anechoic tiny cystic lesions mixed with tubular hyperechoic areas situated along the portal tracts at umbilical portion (*arrowhead*). Because of the cavernous architecture of the cluster of minute peribiliary cysts, it shows hyperechogenicity relative to the surrounding liver. (**b**–**d**) Noncontrast CT (**b**) shows periportal hypoattenuation around the hilar portal veins (*arrow*), which can mimic conditions such as bile duct dilatation and periportal edema. Dynamic contrast-enhanced CT (**c** hepatic arterial dominant phase, **d** portal dominant phase, **e** equilibrium phase) shows a cluster of nonenhancing tiny cystic lesions with thin cyst wall enhancement (*arrowhead*). *Note*: background liver shows multiple nodularity due to alcoholic liver cirrhosis. Gastric varices caused by portal hypertension are also seen (**d**, *arrow*)

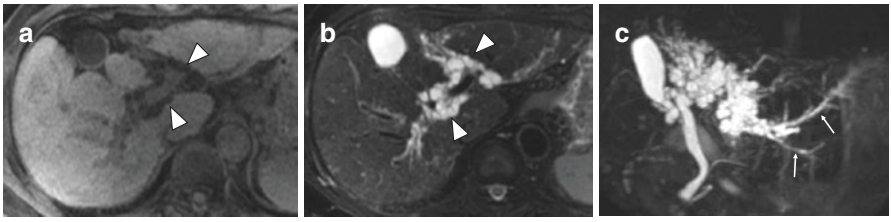


**Fig. 9.4** MRI findings of peribiliary cysts (the same case with Fig. 9.3). (**a**) On T1-weighted image, fluid contents show hypointensity (*arrowhead*). (**b**) On T2-weighted image, fluid contents show hyperintensity (*arrowhead*). *Note*: no peribiliary cysts can be identified in the peripheral intrahepatic bile ducts. (**c**) On MRCP, a cluster of multiple tiny cysts around larger portal tracts is clearly demonstrated. Slight dilatation of peripheral branch of B3 can be seen (*arrow*) caused by compression of bile duct by peribiliary cysts

(Fig. 9.3a). However, a cluster of minute peribiliary cysts often demonstrates hyperechogenicity relative to the surrounding liver because of their cavernous microarchitecture like cavernous hemangioma of the liver. Therefore, peribiliary cysts commonly show a mixture of anechoic and echogenic portions [7, 9].

On noncontrast CT/MRI, larger peribiliary cysts demonstrate density/signal intensity similar to water (Figs. 9.3b, 9.4a, b). When a cluster of minute peribiliary cysts is present, because of the partial volume effect with cyst walls, their density on CT is higher than that of water, and signal intensity on T1-weighted image higher and on

T2-weighted image lower. On CT/MRI they are depicted as tubular lesions along the portal vein or as beaded communicating cystic lesions [9, 19–21]. Characteristically, peribiliary cysts are present on both sides of the proximal intrahepatic portal vein. When they demonstrate only a thin tubular distribution, they may resemble a periportal collar on CT and a periportal abnormal (hyper)signal intensity (PAI) on MRI. In addition, it must be recognized that peribiliary cysts may show a central dot sign on enhanced CT/MRI like that seen in Caroli disease [22]. On dynamic CT/MRI, peribiliary cysts do not show any enhancement except for cyst wall, which demonstrates a faint enhancement, especially in the equilibrium phase (Fig. 9.3b). Therefore, a cluster of tiny peribiliary cysts may be visualized as a faintly enhanced tubular structure (usually heterogeneous) surrounding the portal veins on the late phase of dynamic CT/MRI. On the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI, peribiliary cysts are not opacified in spite of definite visualization of bile ducts indicating no regurgitation of contrast medium (bile) from the parent bile ducts.

MRCP is most precise in the diagnosis of the total picture of peribiliary cysts. It can demonstrate peribiliary cysts as multiple small and tiny hyperintense cystic masses surrounding hilar larger bile ducts [23] (Fig. 9.4c). On the other hand, contrast medium does not flow into peribiliary cysts on ERCP, and a compressive stricture of larger bile duct is not rarely observed (Fig. 9.5). The discrepancy between MRCP and ERCP (and hepatobiliary phase of Gd-EOB-DTPA) in the visualization of peribiliary cyst is useful in the differentiation from Caroli disease.

It is important to differentiate peribiliary cysts from dilated bile ducts, congenital biliary disease such as Caroli disease, periportal edema, cystic neoplasms such as cystic intraductal papillary neoplasm of bile duct (IPNB), cystic metastasis, and abscess on imaging, to avoid unnecessary further examinations and treatments. Among them, dilated bile ducts, Caroli disease, and periportal edema may resemble peribiliary cysts, and the differential points from these three are briefly mentioned above.

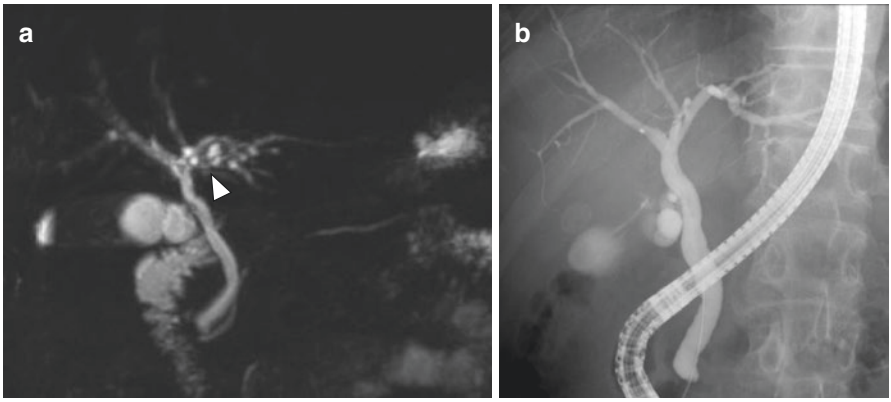


**Fig. 9.5** Peribiliary cysts: comparison of MRCP and ERCP. (**a**) On MRCP, a cluster of tiny cysts can be seen around left large portal tract (*arrowhead*). (**b**) Because of the presence of common bile duct stones in this patient, ERCP was performed. No visualization of peribiliary cysts is seen (no contrast medium influx to peribiliary cyst)

## 9.5 Conclusions

Peribiliary cysts are a common benign cystic lesion. Despite their benign nature, some peribiliary cysts are of clinical significance. Radiologists should be familiar with the imaging variety of peribiliary cysts and differential diagnosis to avoid unnecessary examinations and treatments.

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# Chapter 10
# Immunopathology of Biliary Atresia

**Kenichi Harada**

**Abstract** The obliterative lesion of biliary atresia (BA) is characterized by a progressive sclerosing cholangitis accompanying severe inflammation, fibrosis, and epithelial injuries of extrahepatic bile ducts. Several viral infections including reovirus, rotavirus, and cytomegalovirus and autoimmunity against biliary epithelial cells (BECs) as initial and subsequent events, respectively, have been suspected as a reliable mechanism in the pathogenesis of BA. Biliary innate immunity against double-stranded RNA viruses including reovirus and rotavirus via Toll-like receptor 3 (TLR3) directly induces the dysregulation of the fibrogenic cytokine milieu, biliary apoptosis, and epithelial–mesenchymal transition (EMT). BECs fail to show the innate immune tolerance associated with TLR3 and the activation of biliary innate immunity sustained after a clearance of virus. Although the molecular detection and etiopathic importance of virus in liver and biliary remnants of human BA have been still controversial, the presence of virus could be unnecessary at the subsequent event of acquired immune-mediated bile duct injury. The consequent acquired immunity associated with Th1, Th17, regulatory T cells (Treg cells), and BEC-specific T cells have been clarified, and the immuno-moderation by the regulating of these immunocytes has been noticed as a potential therapeutic target for BA.

**Keywords** Biliary atresia • Innate immunity • Cholangitis • Epithelial mesenchymal transition

## 10.1 Introduction

Biliary atresia (BA) is of two main types, i.e., fetal and perinatal. Fetal biliary atresia (also referred to as prenatal or embryonic type) accounts for 10–25% of all cases of BA, whereas perinatal (or acquired) biliary atresia is the more common type. The fetal type is associated with malformation of extrahepatic bile ducts and other

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concomitant congenital anomalies, which indicates its developmental etiology. The perinatal type is characterized by uncontrolled inflammatory and fibro-obliterative cholangitis in the extrahepatic biliary tree in infancy but is not associated with congenital anomalies. Davenport et al. [1] proposed a new classification of BA into four subtypes: [1] syndromic BA and associated malformations, [2] cystic BA, [3] cytomegalovirus (CMV)-associated BA, and [4] isolated BA.

Irrespective of the subtype, obstruction of extrahepatic bile ducts causes cholestasis causing biliary cirrhosis. Due to the characteristic relentless disease course, untreated BA is a fatal disease and the most common indication for liver transplantation in children. Incidence of BA is approximately 1 in 5000–12,000 live births [2, 3]. In this chapter, immunopathology of BA, especially perinatal type, is summarized.

## 10.2 Etiology of BA

The etiology of BA includes several infectious agents such as viruses and genetic factors such as laterality genes and deranged epigenetic regulation and microRNA function [4, 5]. In the fetal type, genetic abnormalities cause disordered embryogenesis. Several polymorphisms in *HNF6*, *HNF1-B*, *JAGGED1*, and *PKDH1* genes regulating ductal plate remodeling and in *GPC1* that encodes glypican 1, a heparan sulfate proteoglycan that regulates Hedgehog signaling and inflammation, are thought to be as susceptibility factors for bile duct developmental anomaly of BA [6].

The etiopathogenesis of perinatal BA has been well investigated. Several viral infections and maternal microchimerism induce mixed lymphocyte reactions between fetus and the mother [7, 8]. Since the 1980s, several animal models of neonatal hepatitis and cholangitis have been established by Reoviridae (rotavirus and reovirus) infections. In particular, neonatal mice infected by Reoviridae such as type A rhesus rotavirus (RRV) and type 3 reovirus (Abney) is a well-established animal model of BA characterized by cholestasis due to bile duct obstruction [9–12].

Genomes of several viruses including Reoviridae (type 3 reovirus and type C rotavirus), CMV, Epstein–Barr virus (EBV), and human herpes virus (HHV)-6 have been detected in liver tissues and/or bile duct specimens of patients with BA obtained at the time of portoenterostomy operation (Kasai procedure) or liver transplantation. However, some reports have downplayed the pathogenetic significance of these findings [13–21]. Zani et al. [19] recently reported that CMV IgM-positive BA had a poorer outcome, compared with IgM-negative cases, and that the former is a distinct clinical and pathological entity that typically responds poorly to Kasai portoenterostomy. Moreover, in CMV-associated BA, Th1 profile recognized by T-bet expression predominates [22]. Positive immunostaining of hepatocytes and biliary epithelial cells (BECs) for Mx proteins, which are known to mediate early innate immune response and are very sensitive markers for type I IFN activity against viral infection, suggests present and/or previous viral infection in BA [23]. The presence of EBV-encoded RNA (EBER) transcript in hepatocytes and BECs of BA has been demonstrated using an in situ hybridization method [24].

Reoviridae belonging to double-strand RNA (dsRNA) viruses [including reovirus (type 1–3) and rotavirus (A–F groups)] are clinically classified as epithelial tropic viruses. In particular, rotavirus type A is the most common etiological agent in cases of acute infantile diarrhea in young children. In Rhesus rotavirus (RRV)-infected BA mouse models, RRV replication in BECs was shown to mediate the temporal dependence; the degree of injury sustained by immature BECs due to more robust RRV replication correlates with more severe cholangiopathy and is regulated by BEC-producing IFN-α [25]. Human BECs have been shown to be susceptible to RRV infection in a fashion similar to that observed in murine BECs [26]. RRV VP4 surface protein determines BEC tropism, and murine BA model is mediated by RRV VP4-specific activation of mononuclear cells [27]. Although viral particles of RRV are detected in subepithelial cells of RRV-infected BA model [28], it is likely that BECs are also a target of the viruses, directly causing biliary diseases.

Adoptive transfer of hepatic T cells from RRV-infected mice leads to a bile duct-specific inflammatory reaction in recipient mice with severe combined immunodeficiency disease [29]. These findings imply that infections of the biliary tree and subsequent cellular autoimmunity are important for progressive bile duct damage and loss in BA [29, 30]. Regulatory T cells (Treg cells) control the CD8 adaptive immune response at the time of ductal obstruction in experimental BA [31] and also in the conditions of decreased Treg cells of BA infants at the time of diagnosis, T cells specifically reacting to CMV highly detected [32]. However, further studies are needed to elucidate the underlying mechanisms by which these viral infections are linked with the pathogenesis of BA.

## 10.3 Hepatobiliary Lesions in BA

Perinatal BA in the newborn is histologically characterized by progressive fibro-obliterative lesions in the extrahepatic bile duct such as fibrosclerosing cholangitis (periductal and mucosal erosive inflammation) and periductal fibrosis. These changes result in obliteration of the biliary lumen and fibrosis (Figs. 10.1 and 10.2). These characteristic findings still remain in the biliary margins of both duodenal and hepatic sides of the resected biliary fibrous remnant obtained in the Kasai procedure. During the pathogenesis of this ductopenic cholangiopathy, concomitant increase in cell proliferation as well as apoptosis of BECs in the affected bile ducts is found, which suggests that bile duct loss is caused by the imbalanced cell kinetics [33, 34].

In the liver, subsequent cholestasis due to extrahepatic biliary obstruction causes constitutive liver damage and a progressive loss of intrahepatic bile ducts, which eventually progresses to biliary cirrhosis and liver failure in the absence of treatment. The features of peripheral liver in BA are well studied, and a wide variety of histological changes in hepatic tissue of BA have been characterized depending on the state of the disease course at biopsy. In early stages (<3 months of age), biliary

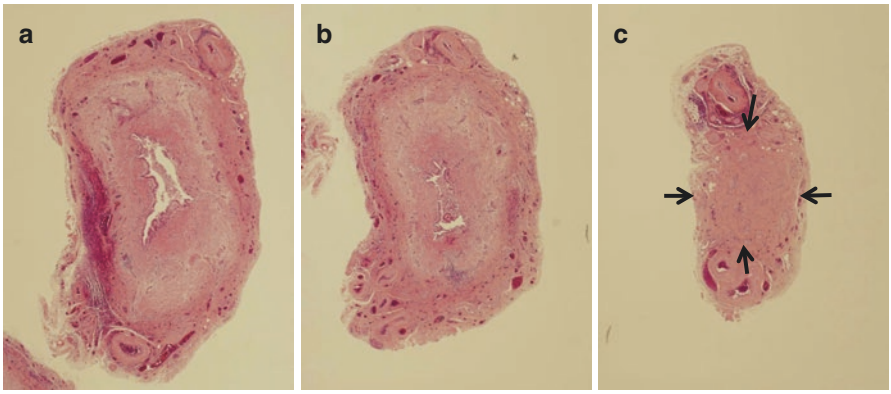


**Fig. 10.1** Transverse sections of extrahepatic bile ducts (biliary remnant) in biliary atresia obtained by Kasai procedure. (**a**) The margin of hepatic side. Biliary lumen is almost preserved, but periductal fibrosis is seen. (**b**) Slit-like narrowed bile duct is seen in adjacent segment of (**a**). (**c**) Atretic bile duct showing luminal occlusion and replaced with fibrous tissue (*arrows*)

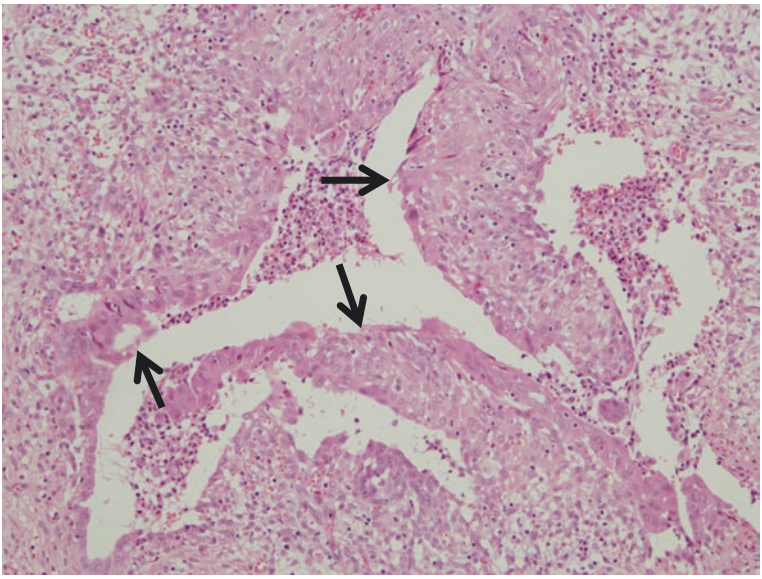

**Fig. 10.2** Transverse section of extrahepatic bile ducts in biliary atresia. Bile duct shows erosive changes, lacks lining epithelia, and shows signs of periductal inflammation. Mesenchymal components consisting of spindle-shaped cells (*arrows*) are found in the surface of bile duct

obstruction-related changes such as ductular proliferation, variable portal edema, lobular cholestasis, and Mallory body are obvious within and around fibrously expanded portal tracts and periportal fibrosis with portal-to-portal bridging are also found. Ductular proliferation with irregular anastomosis resembling ductal plate

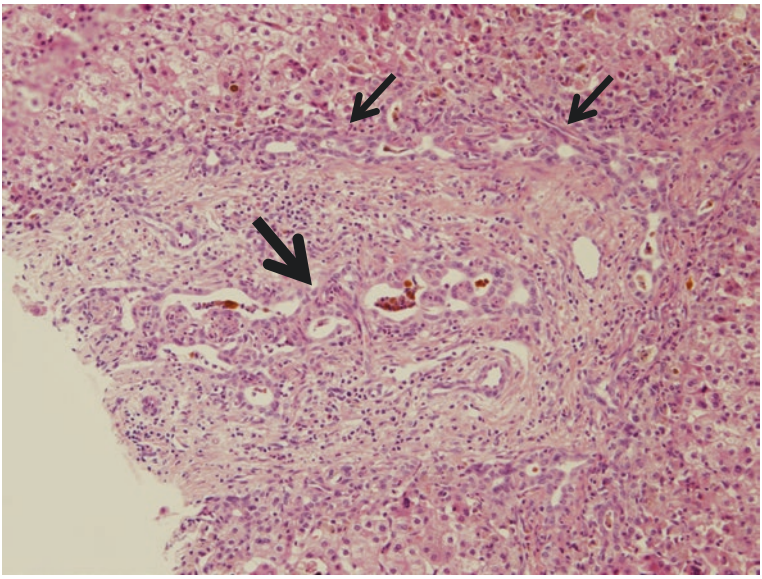

**Fig. 10.3** Portal tracts in biliary atresia. Portal tract is expanded with edematous fibrous tissue and contains irregular anastomosing bile ductules that resemble embryonal ductal plate (ductal plate malformation) (*small arrows*). Distortion and malformation of interlobular bile ducts is also seen (*large arrow*)

malformation is also a well-characterized feature of BA (Fig. 10.3). Nonspecific changes in parenchyma, such as multinucleated giant hepatocytes, extramedullary hematopoiesis, and hemosiderin in Kupffer cells, are also seen. After 3 months of age in the nonoperated patients, the portal fibrosis progresses and further subdivides the hepatic lobule, resulting in biliary cirrhosis. Late stage of BA typically shows less ductular proliferation with marked ductopenia [35].

Recently, several histological indicators of prognostic relevance have been identified in peripheral liver tissues. Duct plate malformation, fibrosis, bile ductular proliferation, cholestasis, hepatocellular alteration, bile duct inflammation, and portal edema have been described as prognosis predictors [36–40]. Obayashi et al. [41] recently proposed that an increased number of biliary elements in portal tracts are a prognostic indicator in BA. The finding was based on an analysis of liver fibrosis, portal–central vein bridging, ductal plate malformation, and the number of the bile ducts and bile ductules in portal tracts. Moreover, a pathological grading system based on two pathological criteria, i.e., bile duct hyperplasia (ductular proliferation) (B1–B3) and fibrosis (F1–F4), was proposed to be useful for prognostic assessment of BA [42]. From the perspective of bile duct repair, sonic hedgehog (SHH) pathway expression in the extrahepatic bile duct of BA has been examined. Strong SHH and glioblastoma-2 (Gli-2) expression was reported to be a poor prognostic factor in Kasai operation-treated patients with BA [43].

## 10.4 Differential Diagnosis

Early diagnosis of BA is of critical import because timely Kasai procedure should be performed for improved biliary drainage, if possible, at an earliest opportunity and preferably within the first 8 weeks of life. However, peripheral hepatic histology of BA is closely similar to and often indistinguishable from those of neonatal cholestasis. It is practically difficult to distinguish preoperatively between BA and other pediatric liver diseases. The differential diagnosis of BA includes other disorders associated with neonatal cholestasis such as neonatal hepatitis, paucity of interlobular bile ducts, alpha 1 antitrypsin deficiency, cystic fibrosis, progressive familial intrahepatic cholestasis (PFIC) type III, and Alagille syndrome [44]. Histopathological findings in neonatal sclerosing cholangitis (NSC), which is caused by mutations in the gene encoding for doublecortin domain containing 2 (DCDC2) protein, closely resemble those of BA [45]. Liver biopsy specimens usually show only intrahepatic lesions related with the obstruction of extrahepatic biliary tract, such as fibrous and inflammatory expansion of portal tracts, marked ductular proliferation, and cholestasis. Although these histological findings are mostly nonspecific in BA, Zheng et al. [46] concluded that the main pathological changes of BA are inflammation and fibrosis in portal tracts. Rastogi et al. [47] evaluated the hepatic histopathology for the presence of features that correlate best with the diagnosis of BA to assess the accuracy of liver biopsy findings. They concluded that ductular proliferation, bile duct and ductular bile plugs, and portal fibrosis are the best indicators of BA, and among these, ductular proliferation is the most important in distinguishing BA from neonatal hepatitis. They indicated percutaneous liver biopsy to be highly accurate (88.2%) in diagnosing BA. Moreover, CD56 immunostaining of liver tissue is a valuable diagnostic aid for differentiating various causes of neonatal cholestasis [48, 49]. CD56, otherwise known as neural cell adhesion molecule (NCAM), is an isoform of neural cell adhesion molecule that is commonly used as a marker of natural killer cells (NK cells) and neuroendocrine cells. Okada et al. [50] reported that immunohistochemical staining of liver biopsy specimen for CD56 is useful in the differential diagnosis of choledochal cyst and type-1 cystic BA (the Japanese Association of Pediatric Surgeons classification [51]) in prenatally diagnosed neonates and that CD56-positive BECs are present in prenatally diagnosed type-1 cystic BA. Zhang et al. [52] also demonstrated that positive expression of CD56 in the immature bile ducts co-expressing notch1 (a key regulatory pathway in bile duct formation) is found in most patients with BA but not in patients with choledochal cysts or neonatal hepatitis. Furthermore, a number of CD56-expressing cells correlated with disease severity, which indicates that maturation of BECs and the expression of notch play a role in the pathogenesis of BA. However, BECs in the proliferating bile ductules expressing CD56 [53] and CD56 staining did not help differentiate BA from other causes of neonatal cholestasis [54]. While several studies, including the above-cited papers, have underscored the utility of liver histopathology, others have pointed out the lack of reliability of the histological diagnosis based on liver biopsy specimens.

## 10.5 Pathogenesis: Innate Immunity

Reoviridae genus (rotavirus and reovirus) show an epitheliotropism and induce apoptosis in intestinal epithelial cells [55]. Reovirus-induced apoptosis is mediated by TNF-related apoptosis-inducing ligand (TRAIL) via activation of the major transcription factor and nuclear factor-κB (NF-κB) [56–58]. These findings suggest that innate immune response against virus could directly cause epithelial insult and cell death. Human BECs possess some Toll-like receptors (TLRs), and the functional expression of TLR1–TLR6 was shown to have a role in the biliary innate immunity [59, 60]. For example, human BECs produce and secrete antibiotics, defensin on stimulation by TLR4 ligand, lipopolysaccharide (LPS), which is the major component of the outer membrane of gram-negative bacteria [61–64]. Moreover, human BECs have TLR3 recognizing dsRNA, which indicates that BECs could directly show innate immune response to dsRNA viruses such as rotavirus and reovirus (Fig. 10.4). Our previous study demonstrated that stimulation with a synthetic analog of viral dsRNA, poly(I:C), induced activation of NF-κB and interferon regulatory factor-3 (IRF-3) and the production of antiviral factor, IFN-β in cultured human BECs [59]. This indicates that BECs have an inherent innate immune mechanism against virus as well as bacteria by production of antibiotics according to the kind of microorganism. The presence of pathogen-associated molecular patterns (PAMPs) inducing innate immune responses as TLR ligands, however, does not always the starting of innate immunity. In any mucosa, mucous layer produced by epithelial cells blocks the direct contact between PAMPs

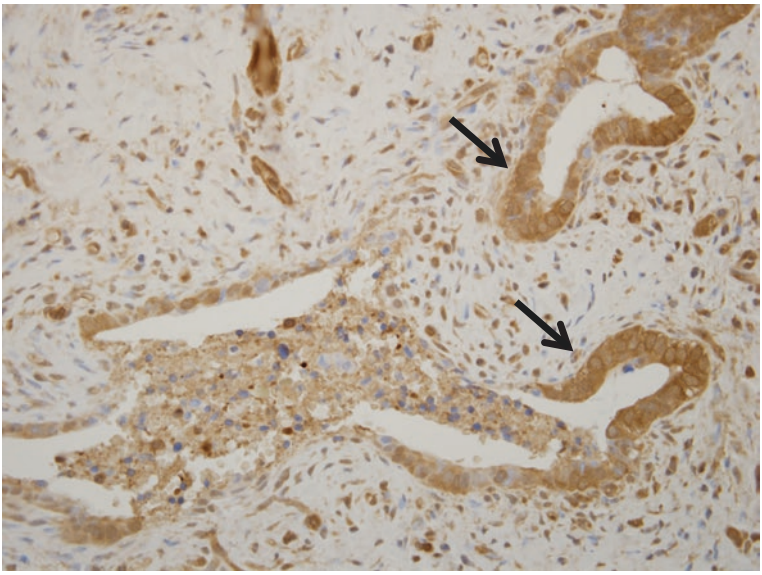

**Fig. 10.4** Immunohistochemical staining for TLR3. Positive expression of TLR3 in the biliary epithelial cells of extrahepatic bile ducts is observed (*arrows*)

and epithelial cells. Moreover, the concomitant inhibitory factors that maintain the tolerant state are expressed, even if innate immune response occurs. As for LPS among PAMPs, the so-called endotoxin tolerance plays a role in avoiding cytokine shock in infectious state. Moreover, the presence of this tolerance mechanism is important to maintain homeostasis of intestinal mucosa and avoid inessential colitis against the intestinal bacterial flora. Human BECs also possess this tolerance system because biliary innate immune response against bacterial PAMPs such as LPS was shown to gradually decrease over time [59]. As a mechanism of this biliary tolerance induction, an inhibitory molecule of TLR intracellular signaling, IL-1R-associated kinase (IRAK)-M is induced by the innate immune response to bacterial PAMPs and is diffusely distributed in the biliary tree to maintain biliary homeostasis in vivo. However, the tolerance against TLR3 ligand, poly(I:C), and also the cross-tolerance between TLR3 and TLR4 are not observed in human BECs [59]. It is reasonable, because this inhibitory site by IRAK-M does not exist in the TLR3 intracellular signaling pathway. The absence of TLR3-related tolerance denotes the absence of tolerance against dsRNA viruses, which indicates that biliary innate immune response and concomitant bile duct damage continues until the complete elimination of dsRNA viruses.

As an important component of the innate immune response, NK cells, NKT cells, and the γδ T cells are immune cell subsets between the innate immunity and adaptive immunity and play a key role in preventing infection, autoimmunity, and tumorigenesis. NK cells play an important part as the first line of defense against several microbial infections, and immune responses from NK- and CD8-positive T cells are involved in the bile duct injury and play a significant role in the phenotype of experimental BA [65]. A population of CD56(−)CD16(+) NK cells having impaired cytolytic functions and cytokine production is expanded as a compensatory measure against the loss of ordinary CD56(+) NK cells. These dysfunctional NK cells may be involved in the retardation of viral elimination and the continuous bile duct damage in BA [66, 67]. However, in a low-dose RRV model of experimental BA, NK cells were shown to promote progressive liver injury and fibrosis and NK cell-depleting strategies were speculated to block progression of liver disease in BA [68]. Moreover, the expression of IL32 ensures continuous inflammation due to increased production of pro-inflammatory cytokines in the damaged bile ducts of BA. Induction of IL32 in cultured human BECs was shown to be induced by biliary innate immune reaction via TLR3 [69]. Immunological disturbances of dysfunctional NK cells in a suspected viral infection and the amplification and continuance of periductal inflammation by IL32 are considered to be key mechanisms that underlie the pathogenesis of cholangitis in BA.

Apoptosis of BECs is speculated to play an important role in the histogenesis of bile duct loss in BA. Increased and disorganized cell kinetics of BECs during liver development are thought to be linked to malformation of the ductal plate and/or abnormal bile duct development [33] and speculated to be caused by impaired expression of E-cadherin in bile ducts [34]. In addition to the production of antiviral factor IFN-β1 through TLR3 signaling, the biliary apoptosis (approximately 30%) is induced by treatment with poly(I:C) via TRAIL production in cultured human BECs [70]. Moreover, an in vivo study demonstrated TRAIL expression in BECs of extrahepatic bile ducts in patients with BA in addition to upregulation of apoptosis and activation of NF-κB and IRF-3, which indicates that in vitro data using human

BECs mimic the histogenesis of biliary lesions in BA and also that the virus infection is directly associated with the cholangiopathy in BA [70]. Because TLR3 is the intracytoplasmic, not membranous, receptor, it is necessary that these viruses directly infect BECs, or these dsRNA genomes are phagocytosed by BECs. In fact, RRV can infect human BECs that result in the production of pro-inflammatory factors such as IL6 and IL8 and profibrotic cytokines via the MAPK pathway [71]. Especially, IL8 is recognized as a critical chemokine and its overexpression positively correlates with inflammation and liver fibrosis in BA [72, 73]. Moreover, Erickson et al. [74] reported early activation of apoptosis in the biliary epithelium and the synergistic role of Th1-related cytokines, IFN-γ and TNF-α, in inducing activation of caspase 3 in BECs in an RRV-infected mouse model. This finding is consistent with a prominent role of apoptosis and Th1-predominant imbalanced cytokine milieu in the cholangiopathy of BA.

As a mechanism of fibrosclerosis, the epithelial–mesenchymal transition (EMT) of BECs is thought to be associated with periductal fibrosis and portal fibrosis in several hepatobiliary diseases including BA [70, 75–79]. Fundamental phenomena observed in EMT include the loss of normal epithelial features such as cytokeratins and cell-to-cell adhesion molecules (e.g., E-cadherin and occludins), gain of a mesenchymal phenotype (e.g., vimentin and smooth muscle actin (SMA)), and acquisition of a fibroblast-like (spindle) morphology caused by cytoskeletal reorganization [80]. The presence of vimentin-positive and epithelial marker-negative bile ducts with activated form of EMT-related transcription factor, Smad3, in extrahepatic biliary remnants of BA (Fig. 10.5) and also α-SMA-positive intrahepatic bile ducts in liver suggests EMT

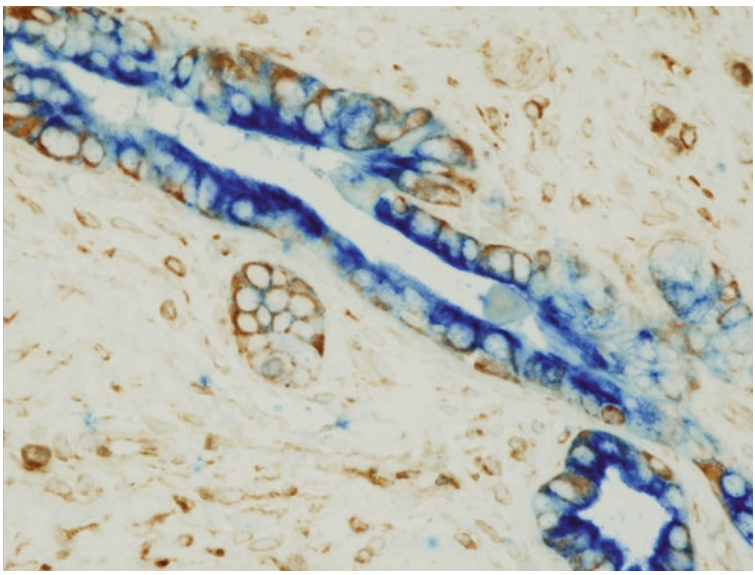

**Fig. 10.5** Simultaneous staining for keratin 19 (*blue substrate*) and vimentin (*brown substrate*). Co-expression of keratin and vimentin in biliary epithelial cells of extrahepatic bile ducts of biliary atresia is observed

of BECs in damaged bile ducts [70, 81]. Transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF) are well-known inducers of EMT [82]. As mentioned above, approximately 30% of cultured human BECs were affected by apoptosis on treatment with TLR3 ligand, poly(I:C), while the remaining 70% cells avoided cell death. By its continuous stimulation in the absence of TLR3-related tolerance, however, the survived BECs undergo EMT via production of bFGF and increased susceptibility to TGF-β1, which suggests the dual presence of biliary apoptosis and EMT in the TLR3-related biliary innate immunity [70]. Biliary innate immune response and concomitant induction of biliary apoptosis and EMT persisted until the complete elimination of dsRNA viruses. As against the acquired immunity, innate immune system exists in epithelial cells as well as immunocytes such as dendritic cells and macrophages. Therefore, human BECs directly prompt the defense response to any infection without any incorporation of immunocytes and probably form the basis of fibrosclerosis and obstruction of extrahepatic bile ducts in BA.

## 10.6 Pathogenesis: Acquired Immunity

In addition to the first-line defense of biliary innate immunity, the consequent acquired immunity is also involved in the pathogenesis of BA. The γδ T cells are unique T cell subsets common to both innate and adaptive immune response and downregulate the immune function by inhibiting $Foxp3^+$ Treg cells. In liver tissues of patients with BA, the increased number of γδ T cells and the inhibition of Treg cell proliferation exacerbate the progressive inflammatory injury of bile ducts [83]. As collateral evidence of the involvement of acquired mechanisms in the pathogenesis of BA, increased levels of serum immunoglobulin, aberrant expression of MHC class II (HLA-DR) and adhesion molecules (ICAM-1 and P-selectin (CD62P)) in damaged bile ducts, and the presence of several pro-inflammatory cytokine-positive activated immunocytes and IL33-positive hepatocytes in liver have been demonstrated in BA [84–89]. Moreover, evidence of oligoclonal expansion of CD4+ and CD8+ T cells in liver tissues and extrahepatic bile duct remnants [90] and that of CD4+ Th1 cell-mediated immunity have been demonstrated in BA. These findings indicate the involvement of Th1-dominat cytokine milieu in the pathogenesis of BA [91–95]. IL17A characterizes the third pathogenic CD4-positive helper T cells, Th17 cells, and promotes various inflammatory and autoimmune processes. Increased γδ T cells producing IL17 in mouse model of BA and upregulation of IL17 in liver tissues from patients with BA have been documented [96]. A human study demonstrated that Th17 cells infiltrate the liver in BA and are associated with worse surgical outcomes [22]. Moreover, the imbalance between Th17 and Treg cells, which is regulated by cytokines (including IL-6), in human BA and RRV-induced experimental murine BA model, was shown to aggravate progressive inflammatory injury at the time of ductal obstruction [97, 98], which indicates that IL17 may serve as a therapeutic target.

In animal models, more detailed experiments associated with acquired immunity and autoimmunity have already been reported. Although the target human antigens of acquired immunity are still unknown, the presence of BEC-specific T cells and both cellular and humoral components of autoimmunity-mediated immune response were demonstrated in RRV-infected mice [29]. Especially, IFN-γ-producing T cells in response to BEC autoantigens, the type I IFN-linked deregulation, and the functional dysregulation of Treg cells are involved in the cholangiopathy of BA [32, 95, 99, 100]. Moreover, Treg-depleted neonatal mouse infected with low-dose CMV exhibited obstructive inflammation in both the intrahepatic and extrahepatic bile ducts that mimics human BA and the involvement of Th1-related cellular and humoral autoimmune responses with the presence of serum autoantibodies reactive to BEC proteins (α-enolase as an autoantigen identified) [101]. B cell-deficient mice with impaired T cell activation and Th1 inflammation, which is attributable to the lack of antigen presentation by B cells, were shown to be protected from biliary obstruction in the rotavirus-induced mouse model of BA. This suggests immunomoderation by inducing B cell hypofunction as a potential therapeutic target for BA [102].

## 10.7 Pathogenesis of Hepatic Fibrosis

BA is characterized by hepatic fibrosis as well as fibrosclerosing lesions of extrahepatic bile ducts. The murine model of BA is also characterized by hepatic fibrosis as well as bile duct lesions [103]. Several fibrogenic factors such as TGF-β, platelet-derived growth factor (PDGF), and connective tissue growth factor (CTGF) are essential for the fibrogenesis of BA liver [104, 105], and DNA hypomethylation of PDGF is also reported as a new candidate in BA pathogenesis [106]. In addition to the expression of CTGF in bile ducts of BA [107], the abundant synthesis of the TGF-β regulating extracellular matrix (ECM), PDGF propagating hepatic stellate cells (HSCs), and monocyte chemotaxis protein-1 (MCP-1) recruiting HSCs are demonstrated in BA [108–111]. Moreover, the imbalance of fibrogenesis and fibrolysis caused by several matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), is believed to be associated in the pathogenesis of fibrosclerosing lesions in BA [91, 110, 112]. A recent study demonstrated the expression of MMP7 in intrahepatic bile ducts, proliferating bile ductules, and periportal hepatocytes subsequent to the effective clearance of biochemical and histological cholestasis following Kasai procedure, which suggests MMP7 may be a potential therapeutic target and a valuable postoperative prognostic tool in BA [113]. Moreover, myofibroblastic differentiation of epithelial–mesenchymal hepatic progenitor cells (HPCs), which express the stem/progenitor cell marker PROMININ-1 (PROM1), was shown to be induced by the TLR3-mediated innate immune response (in part via TGF-β pathway activation) to promote hepatic fibrosis in BA [114, 115].

## 10.8 Conclusion

Infection of several virus species is speculated in the etiology and/or pathogenesis of BA. Although the molecular detection and the etiopathogenic importance of these viruses in liver and biliary remnants of human BA are still controversial or unknown, the presence of virus may not necessarily be involved in the subsequent event of acquired immune-mediated bile duct injury. The keynote of BA studies has shown a recent shift from innate to acquired immunity. Compared with intestinal innate and acquired immunity, biliary immune systems are still under investigation. However, both anatomical characteristics and the innate immunity in the biliary tree are surely associated with the occurrence of biliary inflammation.

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# Chapter 11
# Cholangiolocellular Carcinoma: Is It a Subtype of Cholangiocarcinoma or Combined Hepatocellular Cholangiocarcinoma?

**Fukuo Kondo, Toshio Fukusato, Takuo Tokairin, Koji Saito, and Yurie Soejima**

**Abstract** Cholangiolocellular carcinoma (CLC) is classified into the following two subtypes: (a) pure biliary subtype without hepatocellular carcinoma (HCC) component and (b) hepatobiliary subtype with HCC component. The possibles of cell origin of the former subtype are cholangiole, interlobular duct, and ordinary intrahepatic bile duct (septal or larger duct). The cell origin of the latter type may be HCC. The Japanese General Rules for the Clinical and Pathological Study of Primary Liver Cancer described the features of pure biliary type. Whereas World Health Organization Classification 2010 put an emphasis on the features of hepatobiliary subtype. CLC of the true meaning which must have originated from cholangiole may be identified by the following criteria: the size of the cancer duct (smaller than 15 μm), positivity for c-Kit, and absence of an ordinary ICC component.

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This chapter is partly a revision and a commentary of our former articles [1, 2]

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**Keywords** Cholangiolocellular carcinoma • Combined hepatocellular cholangiocarcinoma • Intrahepatic cholangiocarcinoma • Hepatocellular carcinoma • Interlobular duct carcinoma

## Abbreviations

| | |
|---|---|
| CLC | Cholangiolocellular carcinoma |
| EMA | Epithelial membrane antigen |
| HCC | Hepatocellular carcinoma |
| ICC | Intrahepatic cholangiocarcinoma |
| ILDC | Interlobular duct carcinoma |
| JGR | The Japanese General Rules for the Clinical and Pathological Study of Primary Liver Cancer |
| WHOC | World Health Organization Classification |

## 11.1 Introduction

Cholangiolocellular carcinoma (CLC) is a very unique intrahepatic tumor which has different histological features from ordinary intrahepatic cholangiocarcinoma (ICC) [1–6]. Cancer duct of CLC is significantly thinner than that of ICC. Immunohistochemical positivity pattern for epithelial membrane antigen (EMA) is a membranous pattern (positive staining in the luminal membrane) in CLC, whereas a cytoplasmic pattern (positive staining in the cytoplasm) in ICC [1, 2]. This tumor also shows unique radiological and clinical features. The imaging and macroscopic findings usually show mass formation without dilatation of the peripheral bile ducts, and the lesion is frequently associated with chronic liver disease [7–9]. Because of these unique pathological and clinical features, CLC has recently been classified as a different entity from ICC both in "The Japanese General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Japanese General Rule, JGR)" and in "World Health Organization Classification of Tumors 2010 (WHO Classification, WHOC)" [5, 6]. However, the classification of CLC has been different in JGR and in WHOC. In JGR, CLC is classified as a cancer of non-combined-type primary liver cancer, i.e., a subtype of biliary tumor. Whereas WHOC classifies CLC into a subtype of combined hepatocellular cholangiocarcinoma. Many clinicians and pathologists have been considerably confused by this difference of classifications.

In order to solve the problem of this difference, the authors now explain the true characteristics of various types of CLC lesions based on our former studies [1, 2].

## 11.2 Histological Features of Cholangiolocellular Carcinoma: Comparison with Nonneoplastic Intrahepatic Bile Ducts

### *11.2.1 Classification of Intrahepatic Bile Ducts*

Before describing the characteristics of CLC, reviewing the classification of intrahepatic bile ducts is necessary (Fig. 11.1). Intrahepatic bile ducts are precisely classified according to their size and location. Cholangioles (canals of Hering) are small ducts located in peripheral areas of portal tracts. They do not accompany portal veins or arteries (*black arrows* in Fig. 11.1a, b). These ducts are usually smaller than 15 μm. Interlobular ducts (ILDs) and septal ducts are ducts located in the central area of the portal tract accompanying portal veins and arteries. Ducts with a size of 15–100 μm are classified as ILDs (*blue arrows* in Fig. 11.1a, b), while those with a

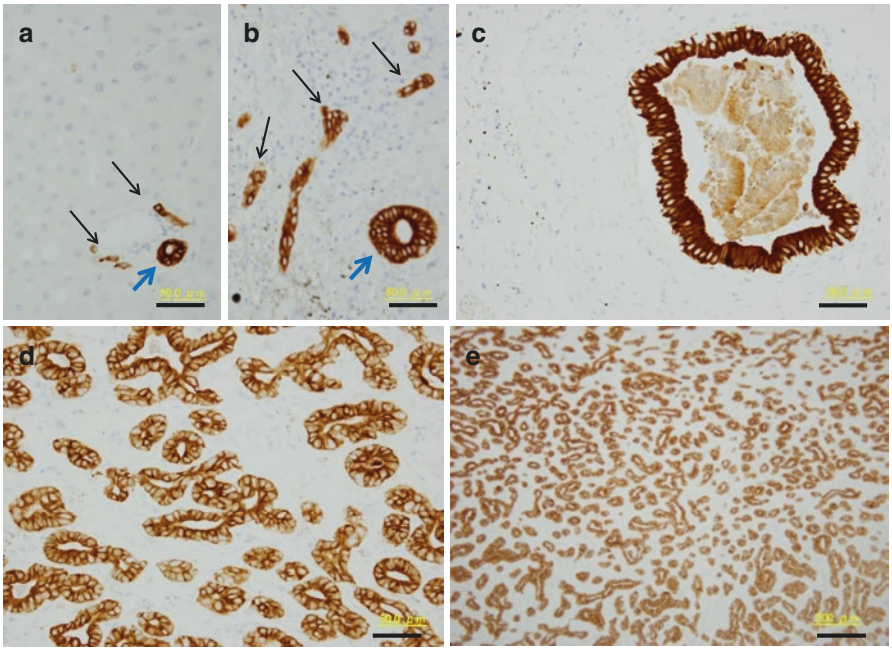


**Fig. 11.1** Comparison of nonneoplastic small bile ducts and CLC (immunohistochemistry for CK7). The *thin black arrows* in (**a**, **b**) show cholangioles. The *blue arrow* in (**a**) shows an interlobular duct of small size (ILD-S). The *blue arrow* in (**b**) shows an interlobular duct of medium size (ILD-M). (**c**) shows a septal duct. (**d**) shows cancer ducts of CLC. Bar: 50 μm (**a**–**d**). In these figures at the same magnification (**a**–**d**), the sizes of the ILD-S, ILD-M, and CLC ducts are clearly larger than that of the cholangiole. However, these ducts are apparently smaller than the septal duct. (**e**) CLC on a low-magnification view. Bar: 200 μm. The duct size of CLC appears to be very thin on this low-magnification view (Adapted from Kondo et al. [1])

size of 100–300 μm are classified as septal ducts (Fig. 11.1c). ILDs are subclassified as ILDs of small size (ILD-S, 15–40 μm) (Fig. 11.1a) and ILDs of medium size (ILD-M, 40–100 μm) (Fig. 11.1b). These bile ducts are collectively called small intrahepatic bile ducts, which are not macroscopically recognizable. In contrast, there exist large bile ducts larger than 300 μm. These ducts are subclassified as third-generation (300–400 μm), second-generation (400–800 μm), and first-generation (>800 μm) ducts [10].

### *11.2.2 Histological Features of Cholangiolocellular Carcinoma*

The histological features of CLC are shown in Fig. 11.1. Cancer ducts of CLC are clearly smaller than septal ducts (Fig. 11.1c, d). On a low-magnification view, CLC ducts appear to be very thin (Fig. 11.1e). However, CLC ducts are apparently larger than nonneoplastic cholangioles when compared in the same magnification (Fig. 11.1a, b, d). Their size is between those of ILD-S and ILD-M.

## 11.3 Classification of Cholangiolocellular Carcinoma

### *11.3.1 Pure Biliary Type*

CLC can be classified into the two subtypes: pure biliary subtype and hepatobiliary subtype (Fig. 11.2). In this meaning, CLC is both a subtype of cholangiocarcinoma and combined carcinoma.

Figure 11.3 shows an example of pure biliary type. The whole area of cancer nodule consists of biliary components. The tumor is negative for Hep Par 1 (Fig. 11.3a) and entirely positive for CK7 (Fig. 11.3b). More than 90% of this tumor showed very thin tubular structure of “antler-like pattern” with abundant fibrous stroma (Fig. 11.1c). Most of the cancer duct showed membranous pattern in EMA immunostaining (Fig. 11.3d). This tumor showed trabecular structure in some areas. Replacing growth was found at its interface with the background liver. However, these features were not recognized as evidence of HCC-like feature of CLC because such findings are also found in ICCs. Mucin was not found in this tumor. These histological features were those of CLC described in the JGR [5]. In JGR, CLC is not classified as combined carcinoma. All CLC lesions in our former study [2] were pure biliary type. In addition to these “pure CLC subtype,” there are some subtypes which include ICC component of various ratio (Fig. 11.2). Because ICC as well as CLC is biliary tumor, these subtypes are “pure biliary subtypes.”

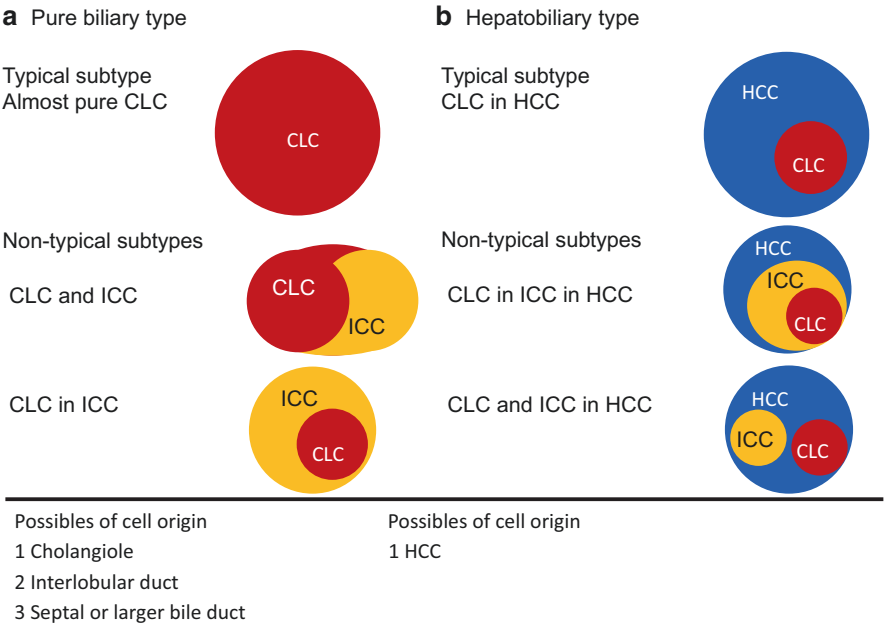


**Fig. 11.2** Classification of cholangiolocellular carcinoma. CLC is classified into two subtypes. (**a**) Pure biliary type without HCC component and (**b**) hepatobiliary type with HCC component. Typical subtype of pure biliary type is almost pure CLC. More than 90% of the tumor is CLC. In addition to this pure CLC, ICC component sometimes coexists in various ratios. Cell origin of these subtypes of pure biliary type can be cholangiole, interlobular duct, and ordinary intrahepatic bile duct (septal or larger bile duct). Typical subtype of hepatobiliary type is "CLC in HCC." ICC components sometimes coexist in various patterns, i.e., "CLC in ICC in HCC" and "CLC and ICC in HCC." Possible cell origin of hepatobiliary type is HCC

### *11.3.2 Hepatobiliary Type*

By contrast, CLC is classified as a subtype of combined carcinoma in WHOC [6]. Figure 11.4 shows typical features of hepatobiliary-type CLC. This tumor consists of HCC component and biliary component, i.e., CLC. HCC component is positive for Hep Par 1 and negative for CK7. CLC component shows reverse pattern. Histologically, HCC component shows ordinary feature of HCC. CLC component shows similar features to pure biliary-type CLC although there exists transitional area between the two components. This nodule-in-nodule pattern (a smaller CLC nodule in a larger HCC nodule) suggests that CLC was formed by transdifferentiation of HCC. Hepatobiliary type may include ICC component in various patterns, namely, "CLC in ICC in HCC" and "CLC and ICC in HCC" subtypes (Figs. 11.2 and 11.5). Figure 11.5 shows "CLC in ICC in HCC" subtype.

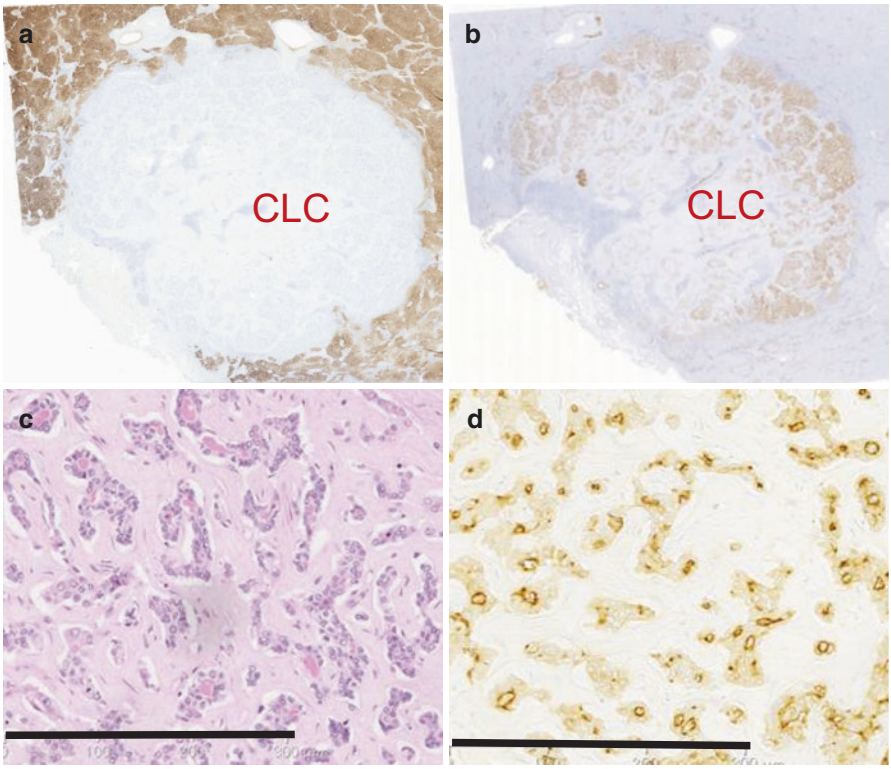


**Fig. 11.3** CLC of pure biliary type. (**a**, **b**) Loupe images of Hep Par 1 and CK7 immunostaining, respectively. Whole area of the tumor is negative for Hep Par 1 (**a**) and positive for CK7 (**b**). (**c**) Histological feature. Very thin cancer ducts with antler-like structures are seen with desmoplastic stroma. (**d**) EMA immunostaining. Cancer ducts showed positivity of membranous pattern. Bar: 300 μm (**c**, **d**)

## 11.4 Cell Origin of Cholangiolocellular Carcinoma

Based on these findings, cell origin and formation mechanism of CLC should be discussed. Although CLC has been speculated as a malignant counterpart of cholangiole where hepatic stem/progenitor cells exist, recent study revealed other possibilities [1, 2]. According to the morphometric and immunohistochemical studies of pure CLC, cancer ducts of CLC were proved to be larger than true cholangioles (Fig. 11.1). They closely resembled interlobular ducts both morphometrically and immunohistochemically [1, 2]. Interlobular duct is now highly speculated as a possible cell origin of pure biliary CLC. ICC can also be a possible cell origin because CLC is also found as a minor component of "CLC in ICC" nodule (Fig. 11.2). It is summarized that interlobular duct, ICC, as well as cholangiole, are now possible cell origins of pure biliary-type CLC (Fig. 11.2). CLC of true meaning which originated from cholangiole can be identified by the following criteria led by our former studies [1, 2].

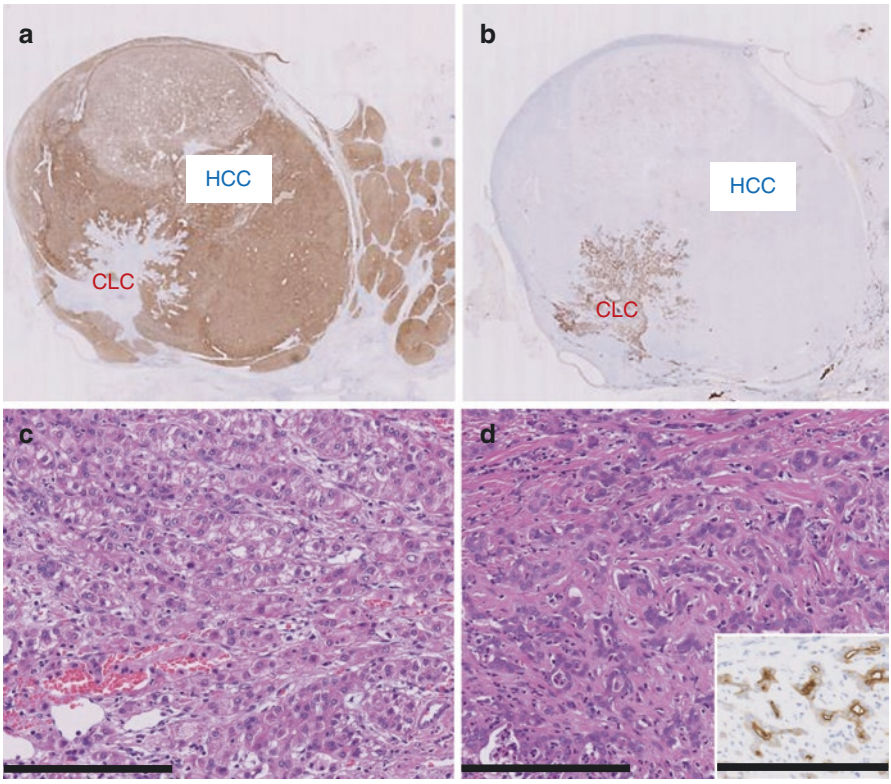


**Fig. 11.4** CLC of hepatobiliary type; CLC in HCC subtype. (**a**) A loupe image of Hep Par 1 immunostaining. Within the large positive area of HCC component, small negative area of CLC is seen. (**b**) CK7 immunostaining. HCC component is negative and CLC component is positive. (**c**) Histological feature of HCC component with typical trabecular structure. Bar: 300 μm. (**d**) CLC component consists of thin cancer ducts with antler-like pattern. Bar: 100 μm. EMA immunostaining. Cancer ducts showed positivity of membranous pattern. Bar: 100 μm. (Through the courtesy of Dr. Takeshi Fujii and Dr. Masafumi Inoue, Department of Pathology, Toranomon Hospital)

1. Cancer ducts are thinner than 15 μm.
2. Cancer ducts show high positive ratio for c-Kit immunostaining.
3. CLC component does not coexist with ICC component.

At present, the tumor which fulfills these criteria is very rare. Many of CLC might have originated from interlobular duct or ICC.

As to the cell origin of CLC of hepatobiliary type, only HCC is speculated (Fig. 11.2). Ordinary HCC tissue without biliary component must have transdifferentiated into CLC in case of "CLC in HCC." In case of "CLC in ICC in HCC," HCC has transformed into ICC, and then ICC must have formed CLC component within the nodule. This formation mechanism may be explained by transdifferentiation theory.

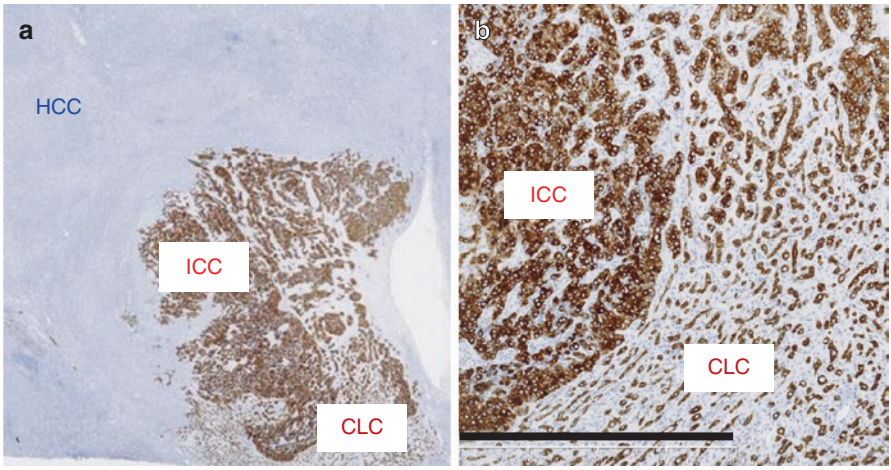


**Fig. 11.5** CLC of hepatobiliary type; CLC in ICC in HCC subtype. (**a**) A low-magnification view of CK7 immunostaining. The largest component of HCC is negatively stained. ICC and CLC components are positive. (**b**) A middle-magnification view. Cancer ducts of CLC are significantly thinner than those of ICC. There is transitional area between ICC and CLC. Bar: 800 μm (Adapted from Shimizu et al. [11])

## 11.5 Stem Cell Characteristics of Cholangiocellular Carcinoma

Because CLC is classified as combined carcinoma with stem cell features [6], the stem cell characteristics of CLC should be discussed according to the classification of this article.

As described formerly, many of CLC of pure biliary type are supposed to be originated from interlobular duct or intrahepatic bile duct (ICC). Only few number of CLC may fulfill the criteria of true CLC whose origin is cholangiole. Even in such true CLC cases, bipotentiality to differentiate into HCC and ICC is not proved in pure biliary type. If true CLC has bipotentiality, small HCC and ICC nodules may be found within a large CLC nodule. At present, we are unaware of CLC lesions with such structure.

In cases of CLC of hepatobiliary type, cell origin is speculated to be HCC. Therefore, stem cell characteristic of CLC is not proved. Even when various stem cell makers, such as CD56, EpCAM, and c-Kit, are positively stained in CLC and HCC areas, it is not necessarily evidence of stem cell origin. These makers are not specific enough to prove stem cell origin and are positively stained in nonneoplastic interlobular ducts [1]. Our recent study suggested that ordinary HCC without ICC component can acquire the characteristic of positivity of cholangiocyte and stem cell markers during the process of tumor progression [12]. Such positivity may be interpreted as “acquired stemness” rather than “primary stemness” (evidence of stem cell origin).

## 11.6 Final Comments

In this chapter, the authors described histological features, classification (subtypes), cell origin (formation mechanism), and stem cell characteristics of CLC. More detailed studies will be necessary to clarify the true characteristics of CLC in the future. The authors sincerely hope this chapter is useful for the understanding of CLC.

**Conflict of Interest Statement** The authors have no conflicts of interest or financial ties to disclose.

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# Chapter 12
# Pathology of Intrahepatic Cholangiocarcinoma: Peripheral and Perihilar Type

**Shinichi Aishima**

**Abstract** Intrahepatic cholangiocarcinomas exhibit histological diversity in terms of morphological architectures, background liver diseases, mucin production, stromal reactions of inflammatory cells or fibrosis, the presence of premalignant lesions, and the coexistence of metastatic lesions. ICCs are classified according to anatomical location and histological characteristics as perihilar large duct type and peripheral small duct type. The different biological and molecular features of the two types of ICC support the hypothesis that perihilar type ICC and peripheral type ICC arise from different backgrounds, different carcinogenic pathways, and different cell origins. It may be important to understand these differences of tumor characteristics for the clinical management of ICCs.

**Keywords** Perihilar cholangiocarcinoma • Peripheral cholangiocarcinoma • Biliary intraepithelial neoplasia • Mucin • Bile duct

## 12.1 Anatomical Classification of Biliary Tract

The biliary tract is a complex network of bile ducts that connects the liver to the duodenum, at the ampulla of Vater. The biliary tract is referred to as the biliary tree because it begins with many small branches of bile ducts that join to form the right and left hepatic ducts in the hilar portion and finally the common bile duct in the extrahepatic portion. Intrahepatic bile ducts are proximal to the right or left hepatic duct and are classified as intrahepatic large and small bile ducts [1]. The intrahepatic large bile ducts consist of the first to third branches of the hepatic bile ducts with peribiliary glands within the duct walls. Intrahepatic small bile ducts consist of septal and interlobular bile ducts without peribiliary glands. The small bile ducts are lined by four to five cholangiocytes characterized by a cuboidal shape with a

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basement membrane and a high nucleus-to-cytoplasm ratio, while large bile ducts are lined by columnar epithelium with relatively small nucleus and abundant cytoplasm [2]. The intrahepatic bile duct system can be divided by duct diameter into hepatic ducts (>800 um), segmental bile ducts (400–800 um), area ducts (300–400 um), septal bile ducts (100–300 μm), interlobular bile ducts (15–100 μm), and small bile ductules (<15 um). Smaller bile ductules connect to the canals of Hering. The canals of Hering are located at the periphery of the portal tracts, and they represent the hepatocyte-cholangiole interface within the lobule [3]. The canals of Hering are not readily apparent on routine histological staining but are highlighted by CK19. The morphological diversity of the biliary epithelium is associated with different functions of cholangiocytes and different biliary diseases.

## 12.2 Anatomical and Gross Classification of Intrahepatic Cholangiocarcinoma

It has been believed that intrahepatic cholangiocarcinomas (ICCs) arise from the epithelial cells of the biliary tree. The clinical presentation of a cholangiocarcinoma may depend on the site of the injury affecting the biliary tract. Involvement of cancer cells in the large bile duct can cause the dilatation of peripheral bile ducts and often lead to obstructive cholestasis. The surgical approach for cholangiocarcinoma also depends on the anatomical site of the tumor. Therefore, it is important to categorize ICCs on the basis of the different anatomic sites where they occur. Okuda K et al. separated ICCs of autopsy cases into the hilar type, which are tumors involved in the hepatic hilum, and the peripheral type, which are tumors occupying the hepatic periphery, and proposed that hilar type ICCs resembled extrahepatic cholangiocarcinoma and that the peripheral type ICCs showed characteristics intermediate between ICC and hepatocellular carcinoma [4]. Hilar cholangiocarcinoma has been defined as Klatskin tumors; however, Nakeeb et al. proposed a definition of the perihilar type as tumors involved in the bifurcation of the hepatic duct or requiring surgical resection of the bifurcation, even if those tumors are located in the liver [5]. Ebata et al. defined the perihilar type as tumors involving the hilar bile duct with a liver mass [6]. Indeed, it is difficult to make clinical and pathological distinctions between cholangiocarcinomas arising from the intrahepatic bile duct and those from the extrahepatic bile duct in the perihilar region.

ICCs can be classified into a mass-forming type, periductal infiltrating type, and intraductal growth type by macroscopic growth patterns. The mass-forming type exhibits well-defined rounded mass formation in the liver parenchyma. The tumor is usually firm and tannish white. The mass-forming type often arises from chronic liver disease of hepatitis or liver cirrhosis. The periductal infiltrating type shows longitudinal tumor extension along the bile ducts involving the connective tissue of the portal triads. Periductal invasion with biliary stricture in the large bile duct may lead to the dilation of peripheral bile ducts. The intraductal growth type proliferates within the lumen of bile ducts like a tumor thrombus. Many intraductal growth type

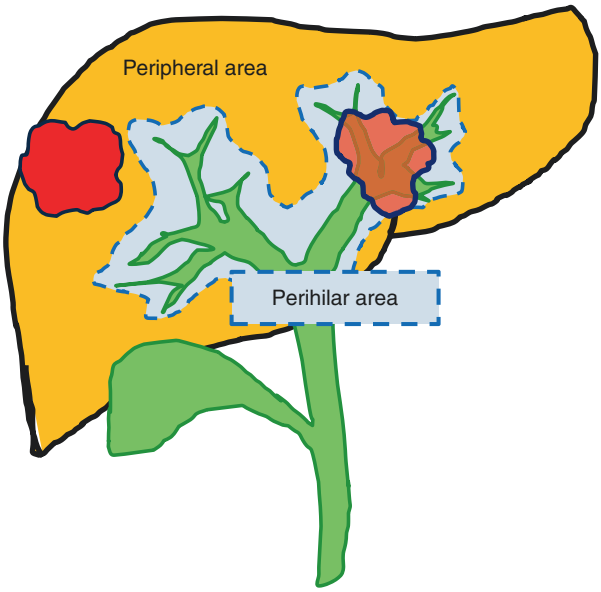


**Fig. 12.1** Perihilar and peripheral areas along the bile ducts of the liver

cases are recognized in the large bile duct, and these share the features of intraductal papillary neoplasm of the bile duct (IPNB). The periductal infiltrative type and intraductal growth type rarely show necrotic or hemorrhagic change. The mixed features of the mass-forming and periductal infiltrating types are associated with jaundice, portal vein invasion, and lymph node metastases, and seem to have a worse prognosis than other types of ICC [7].

Considering the gross patterns and anatomical location of ICC, the mass-forming type tends to be located in peripheral area, while the periductal infiltrating type and intraductal growth type frequently locate in the perihilar area or intrahepatic large bile duct (Fig. 12.1).

## 12.3 Diseases Associated with Cholangiocarcinoma

Cholangiocarcinogenesis appears to be related with biliary inflammatory conditions. Some predisposing factors for cholangiocarcinoma have been identified including hepatolithiasis, primary sclerosing cholangitis (PSC), congenital biliary dilation, liver fluke infection, and pancreatobiliary maljunction. Hepatolithiasis with prolonged infiltration of histiocytes, neutrophils, lymphocytes, and plasma cells, together with calculi, bile juice, or bacterial infection, can induce biliary hyperplasia and neoplastic changes at the bile ducts [8]. PSC is characterized by chronic inflammation, followed by intense periductal fibrosis of the relatively large ducts in the biliary tree. High-grade biliary dysplasia of the large bile ducts was

observed in 83% of patients with PSC [9]. Hepatobiliary fluke infection via the ingestion of raw or undercooked fish is associated with the development of cholangiocarcinoma in Southeast Asia [10]. Congenital dilatation of the bile duct, also called choledochal cyst, is characterized by the cystic dilatation of bile ducts and is associated with pancreatobiliary maljunction [11].

More recent studies have shown that hepatitis C virus or B virus infection and liver cirrhosis may be associated with ICC development [8, 12, 13]. Hepatolithiasis, PSC, and congenital biliary dilatation may contribute to the development of biliary cancer from the large bile duct, while viral infection and liver cirrhosis are the background liver diseases of ICC located in the periphery of the liver. Different preneoplastic conditions are also associated with the different types of ICC. These conditions can induce morphological atypia of the biliary epithelium and the accumulation of molecular abnormalities in the atypical epithelial cells, indicating biliary neoplasia.

## 12.4 Premalignant Lesion of Cholangiocarcinoma

Cholangiocarcinoma has two main types of precursor lesion: microscopic flat or micropapillary epithelial lesions and grossly visible tumor-forming papillary lesions. The flat or micropapillary lesions of the atypical bile duct epithelium have been defined as biliary intraepithelial neoplasia (BilIN) (Fig. 12.2a). In contrast, papillary and/or tubular masses forming preinvasive neoplasms that form macroscopic tumors with cystic dilatation of the bile ducts have been named intraductal papillary neoplasms of the bile duct (IPNB) (Fig. 12.2b). Histological diagnosis of BiliN is important and can be challenging. Carcinogenesis of the biliary tree involves a chronic inflammatory condition, and biliary inflammation makes it difficult to distinguish reactive epithelial change due to inflammation from neoplastic change. A reactive epithelium with cellular atypia will show heterogeneous cell morphology, including atrophic cells, enlarged cells, pencil-like cells, and a

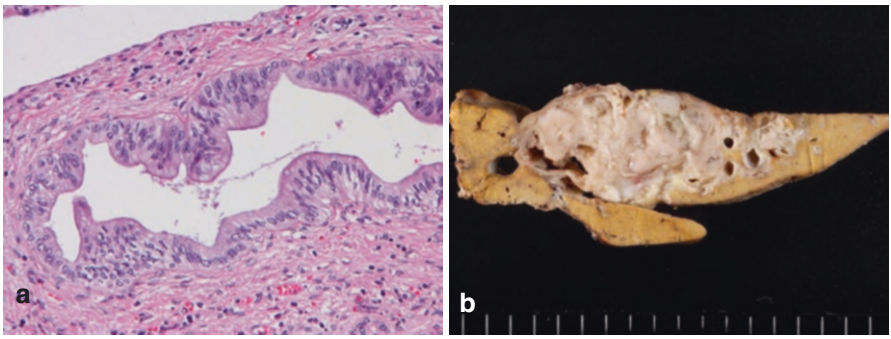


**Fig. 12.2** (**a**) Flat lesions of atypical bile duct epithelium with irregular nuclei defined as high-grade biliary intraepithelial neoplasia (BilIN). (**b**) A grossly mass-forming tumor with cystic dilatation of the peripheral bile ducts is indicative of intraductal papillary neoplasm of the bile duct (IPNB)

cuboidal or columnar epithelium. Distinct nucleoli are occasionally encountered in the reactive epithelium. Biliary neoplastic lesions also arise in cases of liver cirrhosis related to hepatitis B virus or hepatitis C virus infection or alcoholic injury [14]. These studies support the biological plausibility of chronic hepatitis and cirrhosis as potential risk factors for cholangiocarcinoma. Both BilINs and IPNBs are frequently observed in the intrahepatic large bile ducts. Therefore, these two lesions are considered to be precursors of perihilar type ICC, through a multistep dysplasia–carcinoma sequence in the large-sized bile ducts.

## 12.5 Putative Cell Origin of ICC

It had been believed that cholangiocarcinomas arise from cholangiocytes. However, recent studies have identified new insights into the cellular origin of ICCs, indicating that they originate from multiple cell lineages. It has been suggested that stem/progenitor cells of the biliary tree exist at the bottom of the peribiliary glands for the large bile ducts and the canals of Hering for the small bile ducts [15, 16]. Biliary stem/progenitor cells of the peribiliary glands can differentiate into hepatocytes, cholangiocytes, and pancreatic islets, while the canals of Hering can give rise to bipotent cells that are able to differentiate into mature hepatocytes and cholangiocytes and to develop into tumors with hepatocellular and biliary differentiation. Sekiya et al. demonstrated that mature hepatocytes have the potential for transdifferentiation into ICCs through intracellular Notch signaling [17]. These studies suggest that ICC has multiple cellular origins, including mature intrahepatic biliary cells, mature hepatocytes, hepatic stem/progenitor cells, and biliary tree stem/progenitor cells. Considering the existence of two different stem/progenitor cells of the biliary tree, ICCs could be divided into two main forms: tumors arising from the large bile duct with involvement of the peribiliary glands and tumors arising from the canals of Hering or hepatocytes [18].

## 12.6 Classification of Intrahepatic Cholangiocarcinoma

ICCs are a heterogeneous group, and recently several classifications have been reported. Nakanuma et al. divided ICC into conventional ductal carcinoma, bile ductular type, intraductal neoplasm, and rare variants [19]. This classification emphasizes intraductal neoplasms in the view of similarities with pancreatic intraductal papillary-mucinous neoplasm and bile ductular type according to the differences from the conventional type in terms of the origins of the tumor cells. Liau et al. also classified this entity into a bile duct type and cholangiolar type. The bile duct type is composed of tall columnar cells arranged in a large glandular pattern, while the cholangiolar type is composed of cuboidal to low columnar tumor cells that contain scanty cytoplasm [20]. Komuta et al. categorized ICCs as mucin-producing ICCs and mixed-ICCs based on their histological characteristics, and suggested that mucin-producing ICCs

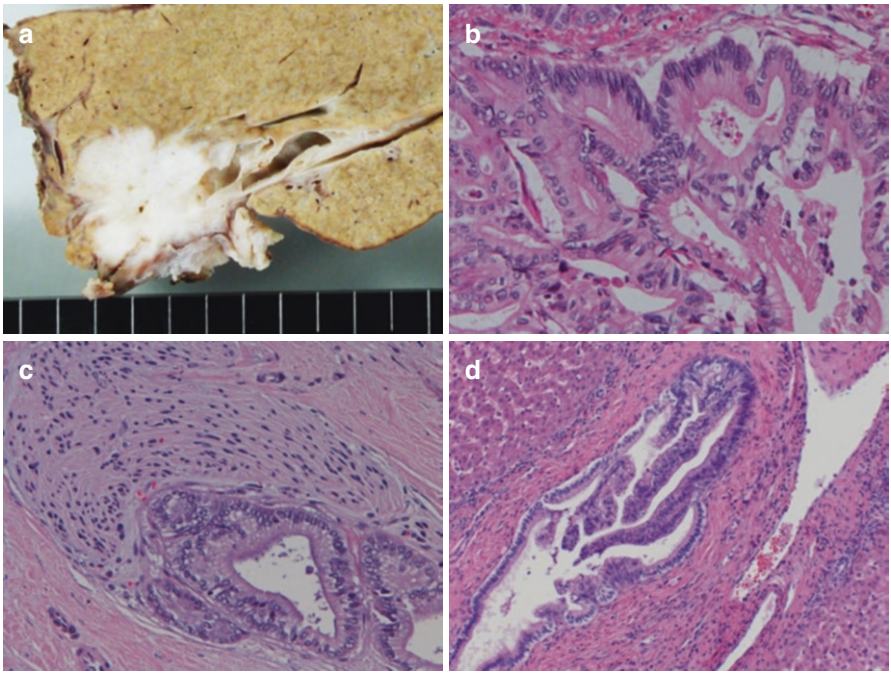


**Fig. 12.3** (**a**) Perihilar large duct type ICC with irregular-shaped mass involved in the left hepatic duct and second branch of the bile duct. (**b**) Atypical tall columnar epithelial cells with mucinous cytoplasm. (**c**) Perineural invasion of large tubular carcinoma. (**d**) Intraductal spreads into the large bile duct

have similarities to hilar CCs, whereas mixed mucin-producing and ductular CCs had a profile similar to that of cholangiocellular carcinoma [21].

ICCs have been classified according to anatomical location and histological features into those involved in large bile ducts, comparable with the intrahepatic second branches, and those involved in smaller-than-segmental branches [22]. The former tumor is perihilar large duct type ICC (Fig. 12.3), suggesting a tumor derived from the large bile duct, and is composed of large tubular components or papillary proliferation of tall columnar epithelial cells (Fig. 12.3b), often admixed with irregular tubular components and poorly differentiated carcinoma cells. Perineural invasion or intraductal spreads of carcinoma are highly observed in the perihilar type (Fig. 12.3c, d). The latter tumor, those involved in the smaller-than-segmental branches, is the peripheral small duct type ICC (Fig. 12.4a), suggesting a tumor possibly derived from the small bile ducts, and is composed of a proliferation of relatively small cuboidal epithelial cells, closely packed, in a cord- or tube-like structure or ductular pattern (Fig. 12.4b), but lacking the large glands by tall columnar cells.

These morphological and histological classifications use different terms; however, perihilar type, conventional ductal carcinoma, bile duct type, and mucin-producing type are in a similar category, while the peripheral type, bile ductular type, cholangiolar type, and mixed or cholangiocellular type are essentially the same kind of tumor.

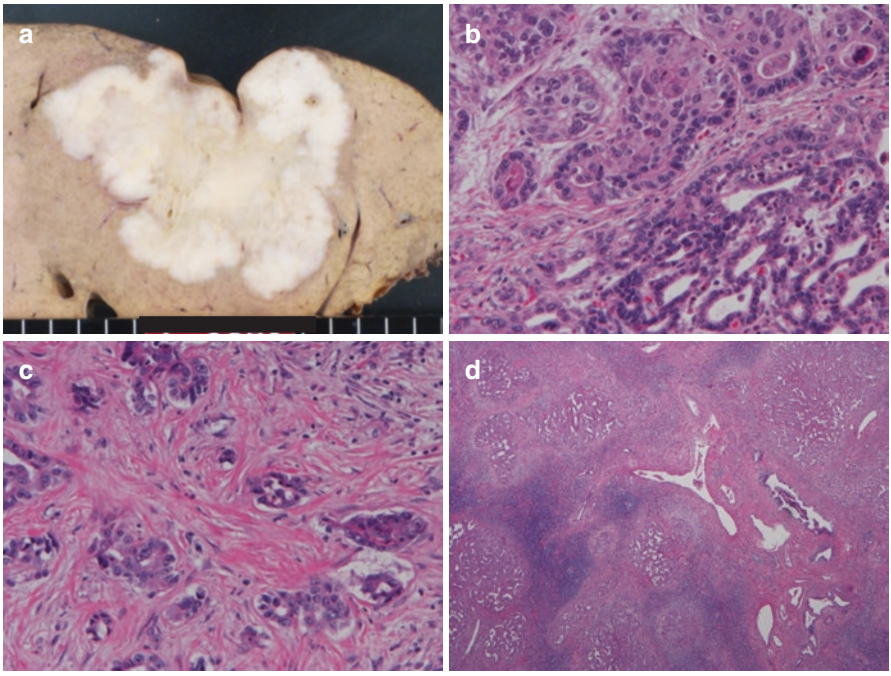


**Fig. 12.4** (**a**) Peripheral small duct type with a firm lobulated mass. (**b**) Small tubules and solid growth by cuboidal and low columnar epithelial cells. (**c**) Scarring stroma with scant atypical glands in the tumor center. (**d**) Large portal triads with islands of cancer nests and inflamed stroma

These classifications are not always applicable to ICCs with large mass formation or ICCs with poorly differentiated histology. In such cases, the detection of the intraductal spreading feature, the presence of BilIN, perineural invasion, and mucin secretion indicate the perihilar type, while the background of chronic hepatic disease, central scarring stroma (Fig. 12.4c), the absence of intraductal spreads or perineural invasion, and preserved architecture of large portal triads within the tumor (Fig. 12.4d) suggest the peripheral type. A recent study suggests that mucin producibility and genetic abnormalities, such as KRAS, isocitrate dehydrogenase (IDH), or fibroblast growth factor receptor 2 (FGFR2) are helpful for ICC classification [23].

## 12.7 Histologic Findings of Perihilar Large Duct Type and Peripheral Small Duct Type Tumors

### *12.7.1 Perihilar Large Duct Type*

Mucin-producing ICCs and IPNBs located in perihilar sites may arise from stem/progenitor cells located in the peribiliary glands of the intrahepatic large bile ducts [15]. Atypical biliary cells frequently develop into BilINs or IPNBs. The periductal

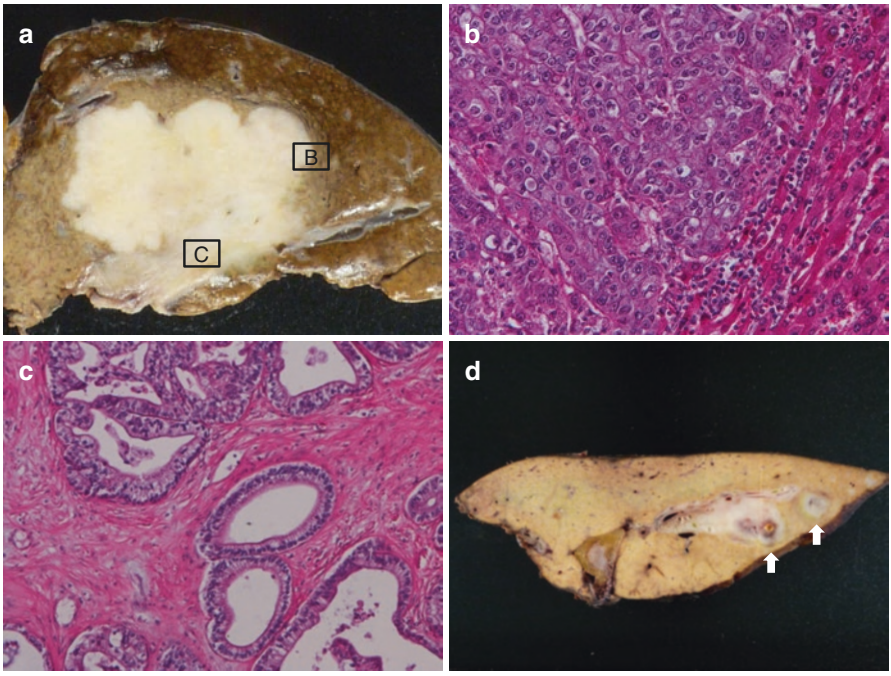


**Fig. 12.5** (**a**) Mass-forming and periductal infiltrating tumor. At first it was unclear whether the tumor was the perihilar type with liver invasion or peripheral type with large duct invasion. (**b**) Histologically, it showed solid proliferation without glandular formation in the tumor border, but was a large tubular carcinoma with columnar cells in the collagenous stroma of the bile duct (**c**), indicating perihilar large duct type with liver invasion. (**d**) There were small whitish lesions with hepatolithiasis in the liver periphery; however, the tumor was found to be large duct type ICC with biliary neoplasia

stromal infiltration of carcinoma cells without parenchymal or vascular invasion suggests early-invasive perihilar large duct type ICCs. These ICCs progress with invasion to the surrounding liver tissue, and spread along the portal tracts results in more advanced stages. Many more lymphatic channels exist in the large portal tracts of the perihilar portion compared with the smaller portal areas. Therefore, lymphatic invasion and lymph node metastasis tend to be observed more frequently in perihilar large duct type rather than peripheral small duct type.

If the tumor is not easily distinguished as a perihilar or peripheral type, especially if it is a mass-forming and periductal infiltrative gross type, mucin production and columnar cell features of cancer cells are important characteristics identifying the perihilar large duct type even in the case of solid growth without definite glandular structure at the invasion site (Fig. 12.5a–c). A few cases of large duct type ICC with hepatolithiasis have been reported in the medium-sized ducts at the liver periphery (Fig. 12.5d). The expression of mucin core protein (MUC) can also be a clue, with MUC2 predominantly expressed in the intraductal growth type [24] and intraductal papillary neoplasm [25] and MUC5AC (gastric foveolar type) frequently

expressed in perihilar type ICC [26]. These findings also suggest that tumor type is related to the potential of mucin production of biliary cell origin.

S100P is a member of the S100 family of EF-hand calcium-binding proteins, and S100P overexpression is a significant characteristic of perihilar ICC [20, 21, 27, 28]. S100P overexpression is also observed in pancreatic cancer and its precursor lesions [29, 30]. Overexpression of CD10 by cancer-associated fibroblasts is associated with invasion and progression in pancreatic cancer [31]. In cholangiocarcinoma, CD10-positive myofibroblasts are predominantly seen in perihilar large duct type ICC and extrahepatic cholangiocarcinoma [32]. These findings indicate that perihilar large duct type ICC has characteristics similar to those of pancreatic cancer from the viewpoint of carcinogenesis and progression [33].

### *12.7.2 Peripheral Small Duct Type*

Mucin hypersecretion is rarely detected in peripheral small duct type ICCs. Interlobular bile ducts may originate from hepatic progenitor cells (HPCs) [18]. Therefore, peripheral small duct type ICCs can be derived from small bile ducts or ductules originating from HPCs. Some small-sized peripheral small duct type ICCs appear to contain preexisting portal tracts with preserved architecture, suggestive of the morphologic characteristics of early-stage peripheral small duct type ICC. Advanced peripheral small duct type ICC shows solid growth of the tumor periphery and extensive fibrotic scarring in the tumor center, with tumor necrosis and intrahepatic metastasis. NCAM is upregulated in mass-forming peripheral type ICC that develops from viral hepatitis (Fig. 12.6a) [34] and with cholangiocellular carcinoma [35]. In such tumors, ill-defined tubular structures resembling ductular reaction are often observed (Fig. 12.6b). Peripheral ICC with viral infection has also been found to express N-cadherin [36, 37]. ICCs with intracytoplasmic hyaline inclusions are frequently of the peripheral mass-forming type with a background of viral infection or alcoholic liver injury (Fig. 12.6c) [38]. ICCs arising in cirrhotic liver or peripheral small duct type ICCs show a higher density of tumor microvessels and arteries [39, 40]. Intratumoral arteries reflect engulfed portal tracts (Fig.12.6d), and decreased arterial vessels indicate aggressive tumor behavior [40].

Gene mutations of isocitrate dehydrogenase (IDH) were reported to be specific alterations in ICC, and these mutations were not detected in extrahepatic bile duct cancer [41]. In a recent study, IDH1 or IDH2 mutations were highly detected in the cholangiolar subtype, consistent with peripheral type ICCs [20].

Cholangiocellular carcinomas show unique histological features, including (1) grossly mass-forming tumors in peripheral locations with typically indistinct tumor borders, (2) antler-like branching morphology of carcinoma cells, (3) cancer cells having round nuclei and a small amount of cytoplasm with fine stroma, (4) no or very weak mucin production, (5) direct contact between the carcinoma cells and hepatocytes at the tumor border, (6) portal tracts or nonneoplastic hepatocyte islands entrapped within the tumor, and (7) cancer cells with eosinophilic cytoplasm,

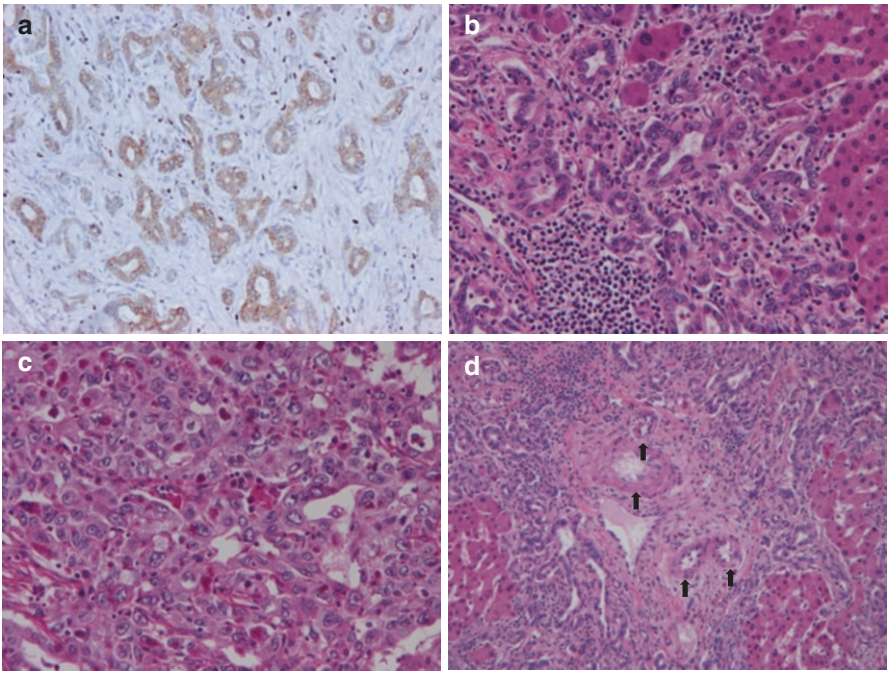


**Fig. 12.6** (**a**) NCAM expression of small duct type ICC. (**b**) Irregular tubular glands with neutrophils replacing the nonneoplastic hepatocytes. (**c**) Ill-defined glandular carcinoma with intracytoplasmic hyaline inclusion. (**d**) The peripheral small duct type ICC contains many arteries

resembling hepatocellular carcinoma [35, 37]. Some cases of peripheral small duct type may be progressive-type cholangiocellular carcinoma because of histologic similarities.

## 12.8 Radiological Findings of Cholangiocarcinoma

According to preoperative dynamic CT findings, if the main tumor location is judged to be at the second or third branch of the intrahepatic bile duct, this case indicates a perihilar location, and if the main tumor location is judged to be at the periphery of the liver, it is described as a peripheral location. Preoperative classification of ICC into perihilar large duct type and peripheral small duct type using imaging findings is important for clinical management. It would be very useful if these two types of ICC could be radiologically discriminated based on the density of intratumoral arteries and the degree of fibrous stroma. Perihilar large duct type ICCs often contain diffuse fibroblastic and collagenous stroma associated with Grissom's fibrous capsules, while peripheral small duct type ICCs show dense distribution of carcinoma cells in the tumor periphery and abundant fibrous stroma in the tumor

center, suggesting hypovascular features by clinical imaging. Enhancement on delayed-phase CT scans is correlated with the amount of fibrous stroma and is a reliable indicator of the prognosis in the mass-forming type [42]. Peripheral small duct type ICCs show a higher density of intratumoral arteries [40]. Hypovascular ICCs in the hepatic arterial phase tend to be of the perihilar type and to have more malignant potential [43]. Cholangiocellular carcinomas have the dual imaging characteristics of hepatocellular carcinoma and cholangiocarcinoma. The absence of a fibrous capsule, the absence of tumor necrosis, the peripheral location in the liver, and the presence of portal venous penetration appear to be characteristic features of cholangiocellular carcinoma [44]. Considering the distinct clinicopathological differences between these two types of ICCs, further imaging studies and pathological classification are needed.

## 12.9 Summary

Evidence-based therapies require the subclassification of various kinds of malignant tumors. Molecular subclasses for hepatocellular carcinomas have been recognized. Although ICC classification has not been fully established, recent studies have identified the molecular abnormalities and histological characteristics specific to different types of ICCs. Understanding the distinct anatomical locations and gross, microscopic, and molecular features of ICCs may improve the use of targeted therapy techniques.

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# Chapter 13
# Intraductal Papillary Neoplasm of the Bile Duct

## A Grossly Visible Preinvasive Neoplasm of the Bile Duct

**Yuki Fukumura, He Cong, Kieko Hara, Yuko Kakuda, and Yasuni Nakanuma**

**Abstract** Intraductal papillary neoplasm of the bile duct (IPNB) is a grossly visible, preinvasive neoplasm of the bile duct that characteristically shows an intraductal predominant growth in dilated bile duct(s). IPNB is composed of a well-differentiated papillary or villous neoplasm covering delicate and ramifying fibrovascular stalks. Tubular components are usually admixed, although they usually constitute less than 50% of the neoplasm. IPNB is regarded as the counterpart of pancreatic intraductal papillary mucinous neoplasm (IPMN). As in IPMN, IPNB is divided into the intestinal, gastric, pancreatobiliary, and oncocytic subtypes and further classified into low-intermediate-grade and high-grade intraepithelial neoplasms. The cases of IPNB with invasion are called "IPNB with an associated invasive carcinoma." IPNBs arising in the intrahepatic bile duct and right or left hepatic bile ducts are quite similar to IPMNs, whereas those arising in the extrahepatic bile duct, particularly in the distal bile ducts, show more aggressive features and more tubular components in comparison to IPMNs. Ordinary cholangiocarcinoma with an inconspicuous or low-height intraluminal papillary component, mucinous cystic neoplasms (MCNs), biliary intraepithelial neoplasia (BilIN), and intraductal tubulopapillary neoplasm should be distinguished from IPNB. Molecular and genetic analyses of more cases of IPNB with reference to IPMN are promising for the evaluation of pathologic and biologic heterogeneities associated with IPNB.

**Keywords** Biliary tree • Intraductal papillary neoplasm • Preinvasive lesion Pancreatic counterpart • Cholangiocarcinoma

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## 13.1 Introduction

Biliary tract carcinomas are intractable malignant tumors that are diagnosed at advanced stages in most patients. Recently, biliary tumors showing grossly visible and preinvasive intraductal papillary or polypoid lesions predominantly growing in the bile duct lumen have been the focus of interests for clinicians and researchers because they resemble pancreatic intraductal papillary mucinous neoplasms (IPMNs) and tubulopapillary neoplasms (ITPN). IPMN and ITPN are known as preinvasive neoplasms of the pancreas. Among these biliary neoplasms, the tumors predominantly growing intraductally showing well-differentiated papillary neoplasms covering delicate and ramifying fibrovascular stalks in dilated bile ducts were defined as "intraductal papillary neoplasms of the bile duct (IPNB)" by the World Health Organization 2010 classification of tumors of the digestive system [1]. IPNB can be histologically categorized into four subtypes and classified into low-intermediate-grade and high-grade intraepithelial neoplasms as for IPMN. When IPNB shows invasion, such cases are called IPNB with an associated invasive carcinoma. IPNBs can develop anywhere along the biliary tree, including the intrahepatic and extrahepatic bile ducts.

Herein, we review and compare IPNB to IPMN and other intraductal biliary neoplasms with an emphasis on their pathologies.

## 13.2 Pathology of IPNB

### *13.2.1 Macroscopic Findings*

IPNBs typically manifest as uni- or multilocular cystic tumors with polypoid masses growing into the lumen of the cystically dilated bile duct(s) and/or grossly visible papillary or polypoid lesions in the dilated bile ducts. The former type is relatively common in the intrahepatic bile ducts, while the latter is common in the extrahepatic bile ducts, particularly in the distal bile duct (Fig. 13.1a–d). These polypoid or papillary lesions are soft and fragile. The heights of papillary or polypoid lesions usually exceed 10 mm; however papillary lesions <10 mm but >5 mm showing similar histologies are also frequently experienced, particularly in the latter. Polypoid masses occasionally extend longitudinally and fill the lumen of the bile duct, exhibiting a cast-like appearance, and IPNB is also occasionally multicentric. Intraductal mucin hypersecretion may occur in about one third of IPNBs (Fig. 13.1b). Although luminal communication of the affected bile duct(s) by IPNB with the neighboring bile duct(s) is generally evident, proving this communication can sometimes be challenging, particularly in cystic-type IPNB.

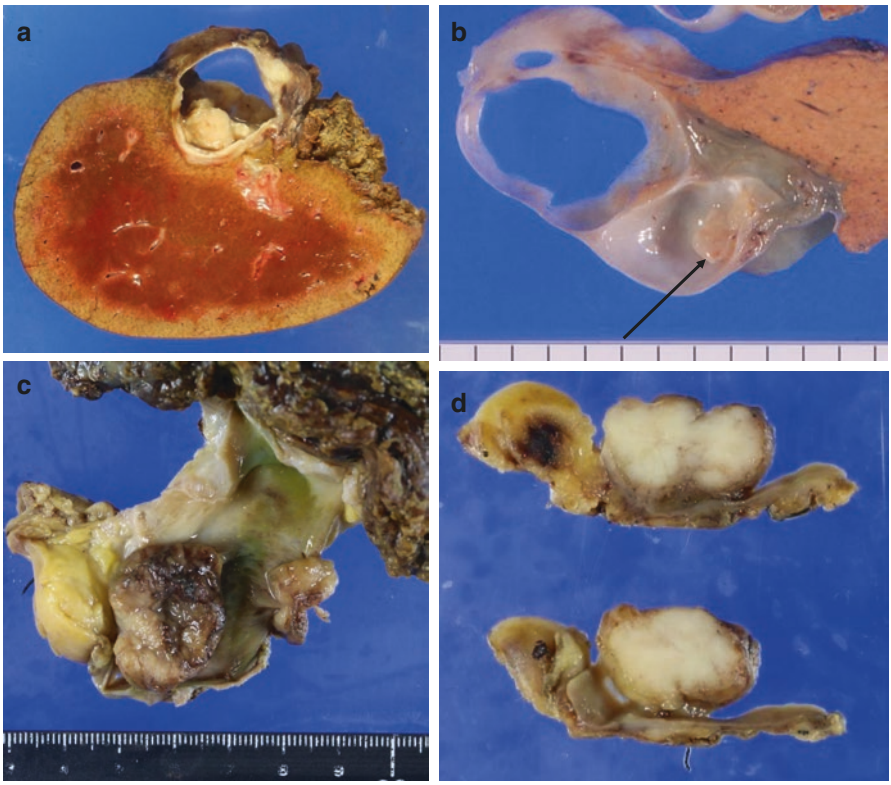


**Fig. 13.1** Gross view of intraductal papillary neoplasm of the bile duct (IPNB). (**a**) A *whitish*, partially lobulated polypoid mass is observable in a dilated right hepatic duct. (**b**) Multicystic tumor with intracystic mucinous nodule (*arrow*) is seen in dilated intrahepatic bile ducts. (**c**) A Polypoid mass is seen in a dilated distal bile duct. (**d**) Cut section of (**c**) shows a *whitish*, lobulated, and partly erosive, polypoid nodule

### *13.2.2 Microscopic Findings*

A predominant intraductal papillary growth of well-differentiated epithelial lining with a delicate and ramifying fibrovascular core is a consistent feature of IPNB. The word "delicate" indicates almost none or only little collagenous stroma in the vascular core (Fig. 13.2). IPNBs are sometimes detected as broad-based papillary masses, sometimes with the circumferential extension of the tumor base, and sometimes as narrow-based tumors. Although the term "tubular" has not been used in the description of IPNB, it often contains a tubular component in addition to a papillary component in the intraductal tumor, similar to IPMN. According to our data, the proportion of the tubular component

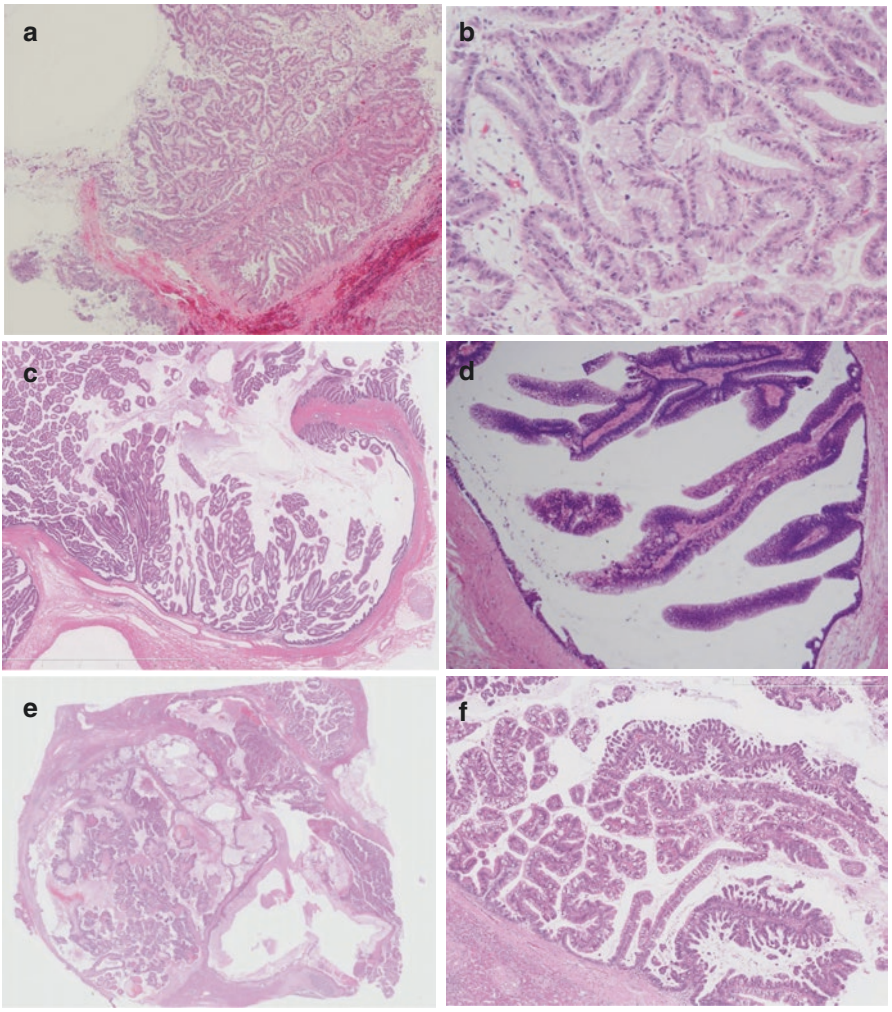


**Fig. 13.2** Microscopic view of intraductal papillary neoplasm of the bile duct (IPNB). (**a**) Gastric subtype (in low-power view); well-differentiated tumor cells grow intraductally with a delicate and ramifying fibrovascular core. (**b**) Gastric subtype (in high-power view); tumor cells with abundant pale mucinous cytoplasm, reminiscent of gastric foveolar/pyloric glands, grow in a papillary/tubular fashion. (**c**) Intestinal subtype (in low-power view); tumor cells grow in tall papilla, reminiscent of colonic villous adenoma. (**d**) Intestinal subtype (in high-power view); tumor cells with pseudostratified, cigar-shaped nuclei and basophilic cytoplasm grow in villous structures. (**e**) Pancreatobiliary subtype (in low-power view); tumor grows in multicystically dilated bile ducts, some with mucin hypersecretion. (**f**) Pancreatobiliary subtype (in higher-power view); tumor cells with variable amount of intracytoplasmic mucin grow in branching papilla. (**g**) Oncocytic subtype (in low-power view); tumor cells grow with edematous, but delicate fibrovascular cores. (**h**) Oncocytic subtype (in high-power view); tumor cells with markedly eosinophilic cytoplasm grow in tubules. (**i**) IPNB of distal bile duct, pancreatobiliary subtype (in low-power view); tumor cells grow in complicated papilla. (**j**) IPNB of distal bile duct, pancreatobiliary type (in high-power view); tumor cells with marked cellular atypia grow in papillary structures accompanied with marked infiltrate of inflammatory cells

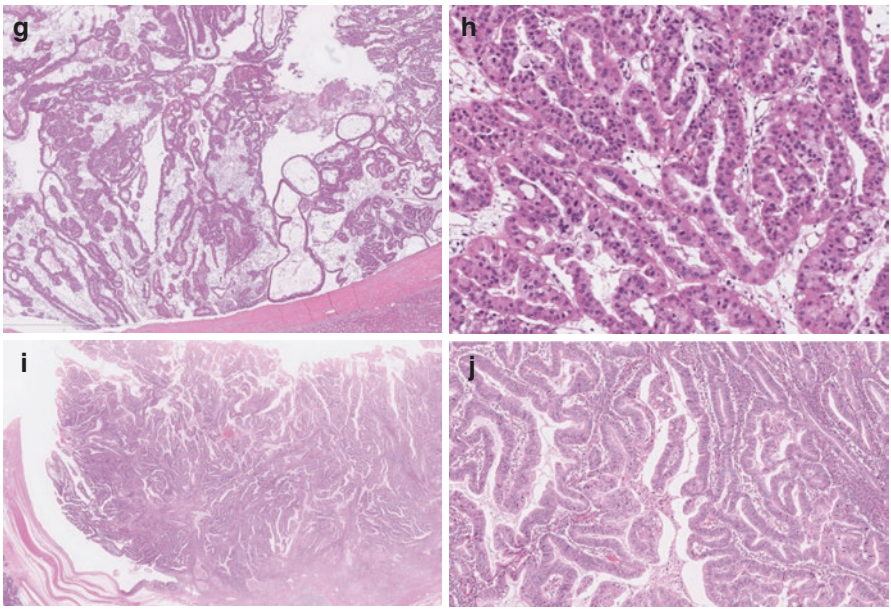


**Fig. 13.2** (continued)

is 0% to ≥50%, and the gastric and oncocytic subtypes often contain plenty of tubular structures (Fig. 13.2a, b, g, h) [2]. However, this tubular pattern of IPNB is different from intraductal tubulopapillary neoplasm of the bile duct (see below). Less than half of IPNB cases show evidence of mucin hypersecretion (mucin-secreting biliary tumors), while mucin hypersecretion is observed in some 80% of IPMN cases (Fig. 13.2c, e) [3, 4].

Upon progression and invasion (IPNB with an associated invasive carcinoma), invasive parts of IPNB manifest as either of tubular carcinoma with desmoplastic reaction, mucinous carcinoma, or even sarcomatous carcinoma, although the frequency and proportion of mucinous carcinoma is much less compared to those for pancreatic IPMN (Fig. 13.3a–d). Some IPNBs show identical histologies to pancreatic IPMNs; that is, by blind observation of the tumor component, the pathologist cannot determine whether the tumor is biliary or pancreatic [2, 11]. However, other IPNBs, particularly arising in the extrahepatic bile ducts, show more heterogeneous histologies, and they are more or less different from IPMNs (Fig. 13.2i, j).

#### 13.2.2.1 Grades

IPNBs can be classified as low-to-intermediate- and high-grade intraepithelial neoplasia based on the degree of cellular/nuclear and structural atypia, and the low- or intermediate-grade category includes borderline lesions, whereas IPNB with the high-grade category with cellular/nuclear and structural atypia adequate

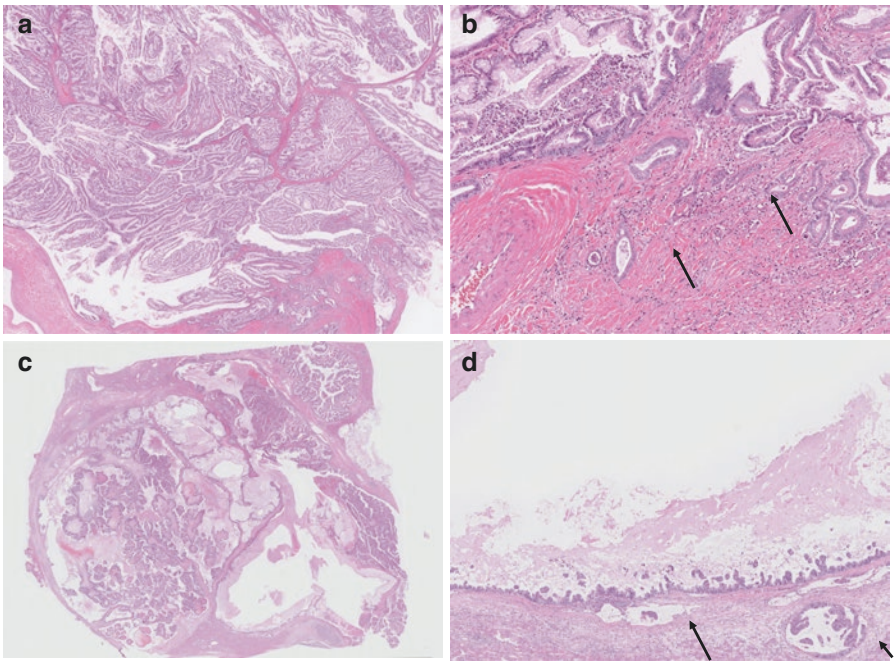


**Fig. 13.3** Intraductal papillary neoplasm of the bile duct (IPNB) associated with invasive adenocarcinoma. (**a**, **b**) IPNB associated with tubular carcinoma. Only a few tubular carcinomas invade into the stroma of intestinal IPNBs with desmoplastic reaction (*arrows*). (**c**, **d**) IPNBs associated with mucinous carcinoma. Several foci of mucinous carcinoma are observed just beside the pancreatobiliary IPNB (*arrows*)

for a diagnosis of malignancy includes "noninvasive" (in situ) carcinomas. IPNB is not infrequently associated with focal and minimal invasion (IPNB with an associated invasive carcinoma) (Fig. 13.3). The proportion of low-grade IPNBs is significantly lower than that of low-grade IPMNs (according to our data, 9.6% vs 38.1%, respectively) [2]. Similar to IPMNs, low-grade intraepithelial neoplasia is most common in IPNBs of gastric subtype, while the other three subtypes more often show more than high-grade intraepithelial neoplasia. In IPNB, two or three grades of intraepithelial neoplasia are not infrequently found in the same case, and in IPNB of high-grade dysplasia with or without invasion, foci of low and/or intermediate neoplasia are also frequently found. This intermingle or mixture of several grades of intraepithelial neoplasm is one of unique and characteristic features of IPNB. Taken together, the entire spectrum of IPNBs represents a continuum of intraductal neoplastic progression and even invasion as proposed in IPMN [5–7].

#### 13.2.2.2 Subtypes

Similar to IPMNs, IPNBs can be classified into the following four histological subtypes using hematoxylin and eosin staining as well as by consideration of expression of phenotypic markers such as MUCs and cytokeratins: intestinal, pancreatobiliary (PB), oncocytic, and gastric. Because the criteria for the subtyping of IPNBs have not been established, the classification is practically performed according to the criteria established for IPMN [8, 9].

The gastric subtype of IPNB is composed of tall columnar cells with abundant pale mucinous cytoplasm reminiscent of gastric foveolar epithelium with/without pyloric-like glands (Fig. 13.2a, b). The intestinal subtype of IPNB is characterized by tall papilla and is highly reminiscent of colonic villous adenomas (Fig. 13.2c, d). The histological double layer structure characterized by gastric foveolar-type epithelia formed at the upper layer and gastric pyloric-type epithelia at the periphery is sometimes observed in IPNB, however, not as frequently as in IPMN. Some IPNBs contain prominent pyloric-like glands similar to some IPMNs (formerly called "intraductal tubular adenoma, ITA"), and such cases are also included in the gastric subtype. The gastric subtype often shows immunohistochemical positivity for MUC5AC/MUC6, but not for MUC1/MUC2.

The intestinal subtype shows intestinal villous patterns and/or goblet cells and often shows positivity for MUC2, MUC5AC, CDX2, and CK20, although the immunoreactivity to these antibodies by the intestinal IPNBs appears to be less stable compared to that for the intestinal IPMNs; MUC2-negative intestinal IPNBs and MUC5AC-negative intestinal IPNBs are sometimes observed [2, 5, 10].

The PB subtype of IPNBs forms thin, branching papillae with a fine fibrovascular core (Fig. 13.2e, f). Generally, the pancreatobiliary subtype itself appears to be heterogeneous with respect to its cellular features and is almost always associated with high-grade dysplasia with or without invasion. It usually expresses MUC1 and MUC5AC.

The oncocytic subtype of IPNBs is characterized by thick branching complex papilla with abundant eosinophilic cytoplasm and hyperchromatic round nuclei (Fig. 13.2g, h). Sometimes, the nuclei are pyknotic. Secondary lumina formation is commonly observed. It usually shows diffuse positivity for MUC5AC and focal expression of MUC1.

In a single IPNB case, more than two subtypes are commonly observed, and the most predominant subtype is regarded as the subtype of individual cases [11]. However, some IPNB cases may have two to four subtypes and thus are difficult to classify as one of the four subtypes. The PB and intestinal subtypes are frequently observed subtypes of IPNB, while the oncocytic ones are rare. The gastric type is infrequent.

## 13.3 Heterogeneities of IPNB

While IPNB is characterized and defined pathologically, its clinical manifestations are variable among individual patients, and its pathological features are also heterogeneous along the biliary tree.

### *13.3.1 Variable Clinical Manifestations of IPNB*

Intraductal papillary tumors corresponding to IPNB have been named by a number of clinical or pathological terms owing to variable clinical manifestations, and this might have caused confusions or difficulties of clinical recognition of these diseases as a single new category and also deferment of therapeutical and research approaches to this disease.

#### 13.3.1.1 Obsolete Pathological Term "Biliary Cystadenoma/Cystadenocarcinoma"

IPNB, particularly that of the intrahepatic bile ducts, shows a cystic dilatation or changes of the affected bile ducts and usually mucin retention. This type of IPNB was previously called as biliary cystadenoma/cystadenocarcinoma [12]. At the moment, the use of cystadenoma/cystadenocarcinoma instead of IPNB is strongly discouraged, and the use of IPNB, particularly cystic IPNB, is strongly encouraged.

#### 13.3.1.2 Obsolete Pathological Term "Biliary Papillomatosis (Papilloma)"

Biliary papillomas and papillomatosis are characterized by single or multiple intraductal papillary tumors with a fine fibrovascular core [13]. While they are well differentiated, some are known to show malignant transformation and even invasion and metastasis. Biliary papillomatosis corresponds to multiple or multicentric IPNB. At the moment, the use of papilloma or papillomatosis is discouraged, while IPNB or multicentric or widely extending IPNB is encouraged.

#### 13.3.1.3 Others

Well-differentiated adenocarcinoma showing papillary growth type of perihilar and distal cholangiocarcinoma (CCA) and that showing intraductal growth type of intrahepatic CCA correspond to IPNB characterized by an intraductal papillary carcinoma with a fine fibrovascular core. The term papillary carcinoma proposed by

Albores-Saavedra also corresponds to IPNBs [14]. Majority of mucin-secreting biliary tumors (MSTB) correspond to IPNB with mucus hypersecretion, though a few cases of MSTB show microscopical or inconspicuous papillary neoplasm in the bile duct lumen. In addition, papillary CCA, mucin-secreting papillomatosis, and papillary adenocarcinoma of the bile duct were also used to refer to IPNB.

### *13.3.2 Pathological Heterogeneity of IPNB Along the Biliary Tree*

To date, majority of clinicopathological studies on IPNBs have mainly focused on those arising in the intrahepatic bile ducts. Recently, there have been several reports predominant intraductal papillary neoplasms of the extrahepatic bile ducts [2, 11, 15]. IPNB arising in the intrahepatic bile ducts tended to present more features similar or identical to IPMN, and the proportion of low-intermediate grade and the frequency of mucus hypersecretion were higher in the IPNB of the extrahepatic bile ducts compared to IPNB of the extrahepatic bile ducts. The incidence of invasion, proportion of tubular components, and the frequency of high-grade intraepithelial neoplasia were higher in extrahepatic IPNB than in IPNB of the intrahepatic bile ducts. The overall pathological features of IPNB of the extrahepatic bile ducts are not similar to those of IPMN. Taken together, the pathological features and aggressiveness of IPNB appear to differ along the biliary tree.

## 13.4 Differential Diagnosis

The following biliary tract neoplasms should be differentiated from IPNB.

### *13.4.1 Conventional CCA with Inconspicuous or Low-Height Intraductal Papillary Components*

In conventional CCA such as nodular sclerosing (NS)-CCA of extrahepatic and perihilar bile ducts and mass-forming intrahepatic CCA, inconspicuous or low-papillary carcinoma components are infrequently found on the luminal surface of the affected bile ducts, in addition to the main or predominant growth and proliferation of tubular adenocarcinoma with desmoplastic reaction in the bile duct wall and periductal tissue. While these inconspicuous or low papillary neoplasms might have preceded the development of conventional CCA, some of these papillary lesions may reflect cancerization of invasive CCA (Fig. 13.4a).

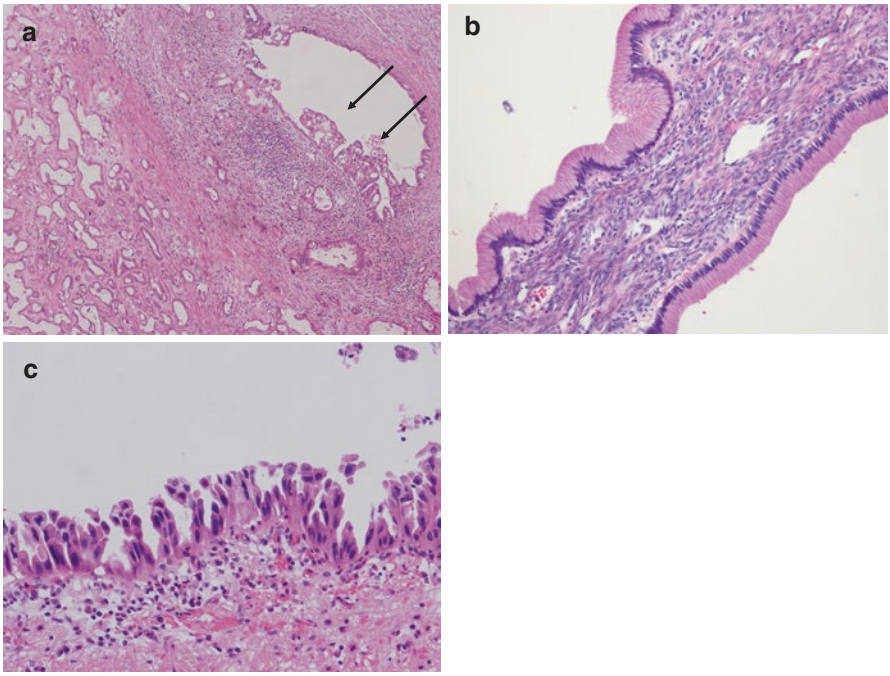


**Fig. 13.4** Biliary tract neoplasms to differentiate from IPNB. (**a**) Conventional cholangiocarcinoma (CCA) with low-height intraductal papillary component. A low-papillary carcinoma component is seen in a bile duct (*arrows*) in conventional CCA. (**b**) Mucinous cystic neoplasm (MCN). A single layer of tumor cells is seen at the cyst wall as tall columnar cells with abundant cytoplasmic mucin. Spindle-shaped stromal cells, so-called ovarian-like stroma (OLS) cells, are observable just beneath the epithelia. (**c**) Biliary intraepithelial neoplasia (BilIN). Low-papillary or micropapillary tumor cells with high-grade nuclear atypia are seen

### *13.4.2 Mucinous Cystic Neoplasms*

When biliary cystic tumors are detected in the liver, we have to think of hepatobiliary (hb) mucinous cystic neoplasms (MCNs) as well. IPNBs, particularly those with cystic dilatation of the affected bile ducts and hb-MCNs, are histologically distinct, when they are typical. Hb-MCNs have densely cellular connective tissue in their wall resembling the ovarian stroma, while this is not seen in IPNBs [3, 16] (Fig. 13.4b). While hb-MCNs predominantly occur in female patients, IPNBs occur slightly more frequently in the male patients. hb-MCNs predominantly occur at the left liver. Usually communication with bile ducts can be observed in IPNBs, whereas this communication is absent in typical hb-MCNs.

### *13.4.3 Intraductal Tubulopapillary Neoplasm of Bile Duct*

Intraductal tubulopapillary neoplasm is a well-established entity in the pancreas [17]. A similar tumor occurs also in the biliary tract and named as intraductal tubulopapillary neoplasm of the bile duct [18]. The tumors were found as a cast-like lesion in the intrahepatic and extrahepatic bile ducts. Histologically, this type of tumor is different from IPNB and shows a predominantly tubular pattern and also solid areas and abortive papillae. Immunohistochemically, these neoplasms were characterized by the expression of MUC1 and MUC6 and by the absence of MUC2 and MUC5AC. Associated invasive carcinomas were present in a majority of cases, mainly conventional tubular adenocarcinoma.

### *13.4.4 Biliary Intraepithelial Neoplasia (BilIN)*

IPNBs and biliary intraepithelial neoplasia (BilIN), which are both precursor lesions of biliary carcinoma, are sometimes difficult to differentiate from each other (Fig. 13.4c). When IPNBs shows flat or low-papillary neoplastic growth, it becomes problematic. Although there are no size criteria for BilIN/IPNB, high-papillary neoplastic growth is usually suggestive of IPNB. When tumor cells show metaplastic changes, BilINs usually show foveolar or pseudopyloric changes, while oncocytic changes are very rare [19].

### *13.4.5 Other Intraductal Growing Tumors*

Several kinds of neoplasms such as signet ring cell carcinoma and sarcoma show predominant intraductal growth.

## 13.5 Conclusion

IPNBs are one of the preinvasive neoplasms of the bile duct and are pathologically characterized by grossly visible, intraductal predominant papillary neoplasm with delicate and ramifying fibrovascular cores covered by well-differentiated lining epithelia. They can be classified into four subtypes (gastric, intestinal, PB, and oncocytic types) and three grades (low-intermediate-grade and high-grade intraepithelial neoplasia). When IPNB show invasion, such cases are called IPNB with an

associated invasive carcinoma. IPNB may present different clinical manifestation among individual patients and show different pathologies along the biliary tree. IPNBs have many characteristics in common with the pancreatic IPMNs; however, they show high heterogeneity in terms of histological and immunohistochemical behavior. Particularly, IPNBs arising in the extrahepatic bile ducts sapper to be different from IPMN in pathological aggressiveness and different grades and subtypes. It is imperative to accumulate scientific evidences and establish distinctive criteria for IPNB in the near future.

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# Chapter 14
# Cystic and Micropapillary Neoplasm of Peribiliary Glands: Its Perspective to Cholangiocarcinogenesis

**Yasunori Sato**

**Abstract** Peribiliary glands are located around the extrahepatic and perihilar bile ducts and are reportedly involved in the development and the diseases of the hepatobiliary and pancreatic systems. In the glandular system, several pathological changes such as cystic dilatation, hyperplasia, and neoplasia have been identified. Cystic dilatation of microscopic size is not uncommon in the peribiliary glands. Rarely, cystic changes are accompanied by micropapillary epithelial proliferation within the glands. The cystic and micropapillary lesions of the peribiliary glands may have neoplastic features, and histological pictures resemble to those of branch-type intraductal papillary mucinous neoplasm of the pancreas other than the smaller size of ductal diameter. They may be precursors of biliary epithelial neoplasms involving intraductal papillary neoplasm of the bile duct as well as cholangiocarcinoma. Although the clinical significance of cystic and micropapillary lesions of the peribiliary glands remains uncharted, the recognition of the lesion seems to be important in biliary tract pathology when the biology and oncogenesis of biliary epithelial neoplasms are considered.

**Keywords** Peribiliary glands • Peribiliary cysts • Micropapillary proliferation • Neoplasia • Oncogenesis

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## Abbreviations

IPMN Intraductal papillary mucinous neoplasm
IPNB Intraductal papillary neoplasm of bile duct
PanIN Pancreatic intraepithelial neoplasia

## 14.1 Anatomy of Peribiliary Glands

Peribiliary glands are located along the biliary tract from the hepatic hilum to ampulla of Vater. They are histologically divided into two types: intramural glands and extramural glands [16, 17]. Intrahepatic peribiliary glands are known to develop from embryonic ductal plate cells [28].

Intramural glands are sparsely distributed within the ductal wall. They are simple tubular glands with few or no branches and are composed of cuboidal to columnar epithelial cells with mucin-filled cytoplasm and basally located nuclei (Fig. 14.1a). They drain directly into the lumen of the bile duct. Under pathological conditions such as chronic cholangitis, these glands are observed to be readily increased.

Extramural glands are located outside the ductal wall. They show anatomical variations in their distribution and density in the biliary tract, distributing more frequently around the hepatic hilum, ampulla of Vater, and cystic duct. They are composed of several lobules of serous and/or mucinous acini in a variable proportion (Fig. 14.1b). The glands are connected through the excretory duct to the bile duct. The adjacent extramural glands frequently anastomose to each other or form a plexus.

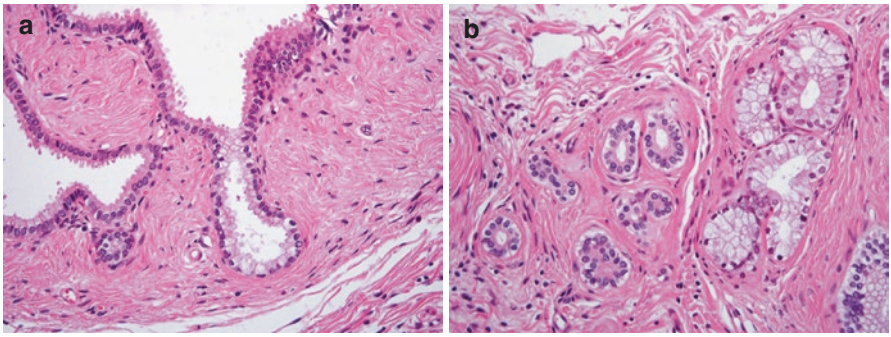


**Fig. 14.1** Histology of peribiliary glands. (**a**) Intramural glands. (**b**) Extramural glands composed of mucinous acini (*right*) and serous acini (*left*). Hematoxylin and eosin staining

These glands, particularly the extramural glands, are supplied by a vascular plexus probably derived from hepatic arteries. There must be active exchange of substances between acinar cells and their adjoining capillaries. A majority of these vessels may drain into portal veins.

## 14.2 Physiology of Peribiliary Glands

### *14.2.1 Presumed Functions*

There are several presumed physiological functions of the peribiliary glands. The intramural glands contain mainly neural mucin, and the extramural glands contain neural and/or acid mucin in a variable combination. The mucin is secreted into the bile and then may act as a lubricant for the bile stream which may also be involved in the self-protective function of the biliary tree.

Immunoglobulin A, lactoferrin, and lysozyme are immunohistochemically demonstrable in the peribiliary glands. These proteins may contribute to local defense against bacterial infection preserving local sterility of the biliary tree.

Extramural glands contain several enzymes for digestion of protein and lipids such as amylase, trypsin, and lipase. Pancreatic exocrine acini are occasionally admixed with extramural glands. Neuroendocrine cells and hepatocyte-like cells can also be found within the acini, where their role remains uncharted.

### *14.2.2 Biliary Tract Stem Cells*

Recently, the peribiliary glands receive much attention as a reservoir of biliary tract stem cells [3, 4]. On immunohistochemical staining, cells within the peribiliary glands are phenotypically heterogenous, and subpopulations of the cells co-expressing stem/progenitor markers of liver (SOX17) and pancreas (PDX1) are assumed to be the most primitive of the biliary tree stem/progenitors [5]. They are located at the bottom of the peribiliary glands and are likely to be central to normal tissue turnover and injury repair and to be key elements in the pathophysiology of the hepatobiliary diseases [8, 9].

The biliary stem/progenitor cells are composed of multiple subpopulations with traits suggestive of maturational lineage stages and yet capable of self-replication and multipotent differentiation, being able to differentiate to mature liver cells (hepatocytes, cholangiocytes) and mature pancreatic cells (including functional islet endocrine cells) [2]. Pancreatic duct glands can be considered as the anatomical counterpart of the peribiliary glands [7]. Similar to the peribiliary glands, the

pancreatic duct glands have been shown to be novel niches containing cells with multiple phenotypes of committed progenitors. They may be important for pancreatic epithelial regeneration [30].

## 14.3 Reactive Conditions of Peribiliary Glands

The peribiliary glands are affected under certain pathologic conditions of the hepatobiliary system. Several reactive conditions of histological changes such as necroinflammation, cystic dilatation, and epithelial hyperplasia have been identified in the glandular system [16, 17].

A necroinflammatory process (adenitis) is occasionally observed in the peribiliary glands. In a series of consecutive autopsied livers, the incidence of adenitis is reported to be 23% 25]. Necroinflammation is associated with biliary tract diseases and chronic advanced liver diseases and may also appear in the livers of subjects with extrahepatic diseases such as sepsis. The adenitis is frequently associated with cystic changes of the peribiliary glands, suggesting that the cystic change may occur as the results of inflammatory destruction of the glandular conduits.

### *14.3.1 Peribiliary Cysts*

Peribiliary cysts are firstly described in the literature in 1984 [15]. Cystic dilation is not uncommon and is observed in 20% of autopsied livers, although a majority is found incidentally at surgery or autopsy and is identifiable only under a microscope [24, 25]. Some glands dilate to a grossly recognizable size, and it is usually less than 2 cm in its greatest diameter. Enlarged peribiliary cysts can compress the adjoining ducts, followed by the biliary compression and dilatation of the proximal intrahepatic bile ducts.

The cysts are usually unilocular, round, thin-walled, and multiple and contain serous fluid (Fig. 14.2a). Histologically, dilated cysts are lined by a cuboidal to

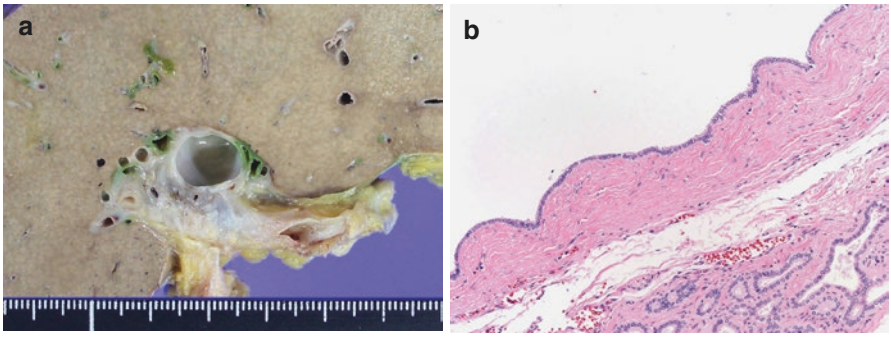


**Fig. 14.2** Peribiliary cyst. (**a**) Grossly visible cyst in the hepatic hilum. (**b**) Histology of the lesion. Hematoxylin and eosin staining

columnar epithelia surrounded by thin fibrous tissue (Fig. 14.2b). Some cyst epithelia are mucin-positive. They are characteristically admixed with non-dilated or mildly dilated extramural glands and their conducts.

Cystic dilation of the glands is shown to be associated with hepatobiliary conditions such as extrahepatic portal obstruction, non-cirrhotic portal hypertension, adult polycystic disease, cirrhosis, hepatocellular carcinoma, and septicemia [24, 25]. The cystic dilatation in portal hypertensive liver diseases may be a manifestation of biliary system alteration occurring in association with a disturbed intrahepatic microcirculation. In addition, hypersecretion associated with hyperplasia of the glandular cells may be followed by the cystic dilatation of the glands.

Peribiliary cysts are likely to occur in chronic alcoholics [11]. The frequent association of peribiliary cysts with alcohol-related hepatic fibrosis suggests the involvement of hepatic fibrosis in the formation of peribiliary cysts. The hepatic fibrogenic process may relate to cyst formation as well as adenitis of the peribiliary glands in chronic alcoholics.

### *14.3.2 Epithelial Hyperplasia*

Hyperplasia occurs in the intramural glands and the extramural serous and mucinous acini [26]. Two or more of these three hyperplastic changes occasionally coexist in an individual liver. They can be seen in various hepatobiliary diseases and even in the normal liver. In hepatolithiasis, it is well known that the intramural and extramural glands proliferate markedly in association with fibrosis and inflammatory cell infiltration (chronic proliferative cholangitis).

The glandular hyperplasia may be associated with seromucinous hypersecretion which leads to biliary dysfunctions such as retardation of bile flow and increased bile viscosity. Because mucinous glycoprotein, particularly when acidic, is a promoting factor in the formation of calcium bilirubinate stones, the glandular hyperplasia with mucinous hypersecretion may be a trigger to hepatolithiasis.

## 14.4 Neoplasia of Peribiliary Glands

Neoplasia may arise from the peribiliary glands. Although their incidence is not so frequent, the peribiliary glands and their conduits show variable atypical hyperplasia as well as papillary hyperplasia, which may represent a preneoplastic lesion or a precancerous lesion of cholangiocarcinoma.

### *14.4.1 Atypical Epithelial Lesions*

According to the previous report, atypical epithelial lesions in the extramural glands can be histologically divisible into papillary hyperplasia, atypical hyperplasia, and carcinomatous transformation [24, 25].

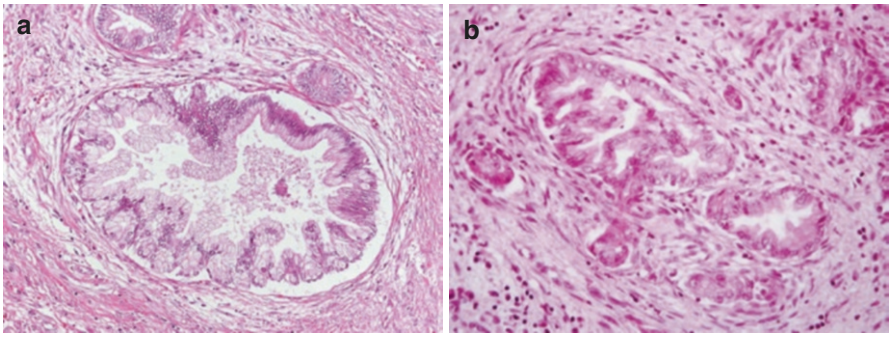


**Fig. 14.3** Atypical epithelial lesions of peribiliary glands. (**a**) Papillary hyperplasia. (**b**) Atypical hyperplasia. Hematoxylin and eosin staining

Papillary hyperplasia consists of epithelia showing tall columnar cells with basally situated nuclei (Fig. 14.3a). The epithelial often project into the glandular lumina accompanying thin fibrous cores. The epithelial cells rarely show atypia such as nuclear hyperchromasia and irregularity. The histologic pictures of papillary hyperplasia of the peribiliary glands closely resemble to those of pancreatic intraepithelial neoplasia-1 (PanIN-1).

Atypical hyperplasia is characterized by epithelial cells with nuclear enlargement, hyperchromasia, and irregularity (Fig. 14.3b). Epithelial stratification and loss of nuclear polarity are occasionally observed. They often show structural abnormalities such as piling of nuclei, luminal papillary projections without fibrous cores, and intraluminal bridge formation. Atypical hyperplasia usually coexists with papillary hyperplasia. Both of papillary hyperplasia and atypical hyperplasia show a mild to moderate dilatation of glandular lumina.

Carcinomatous transformation is a cluster of peribiliary glands with carcinomatous change and slight invasion into the peribiliary connective tissue. The carcinoma is a well-differentiated tubular adenocarcinoma with significant cytological atypia. Mucinous and atypical hyperplasias are present in the vicinity of the carcinomatous area.

### *14.4.2 Cholangiocarcinoma*

Cholangiocarcinoma arising from the peribiliary glands appears to actually exist [27]. Biliary tree stem/progenitor cells located in the peribiliary glands are associated with mucin-producing cells within the liver and biliary tree, and it has been proposed that mucin-producing cholangiocarcinoma may arise from the biliary tree stem/progenitor cells of the peribiliary glands [3, 4]. In response to injuries, pancreatic duct glands undergo a mucinous metaplasia that may lead to

pancreatic cancer, and this could occur also in the biliary tree during pathologic processes with risk factors for cholangiocarcinoma such as primary sclerosing cholangitis [6].

An extensive carcinomatous involvement of the peribiliary glands and their conduits has been reported [21]. These glands and conduits may also give rise to routes by which carcinoma can spread along the biliary tree, presenting a unique form of periductal spread of cholangiocarcinoma.

## 14.5 Cystic and Micropapillary Neoplasm of Peribiliary Glands

Microscopic cystic lesions of the peribiliary glands as well as the peribiliary cysts are usually covered by flattened cuboidal to columnar epithelia. When preneoplastic or neoplastic epithelia are present in the peribiliary glands, they may show histological appearances of either cystic dilatation or micropapillary epithelial proliferation as a result of increased cell proliferation. Although it is a rare lesion, the peribiliary glands accompanied by both cystic change and micropapillary epithelial proliferation can be encountered (Fig. 14.4a).

### *14.5.1 Frequency*

The author has previously surveyed such cystic and micropapillary lesions of the peribiliary glands using a total of 938 autopsy livers [22. In the analysis, the dilatation of the peribiliary glands more than 2 mm in diameter was defined as cystic

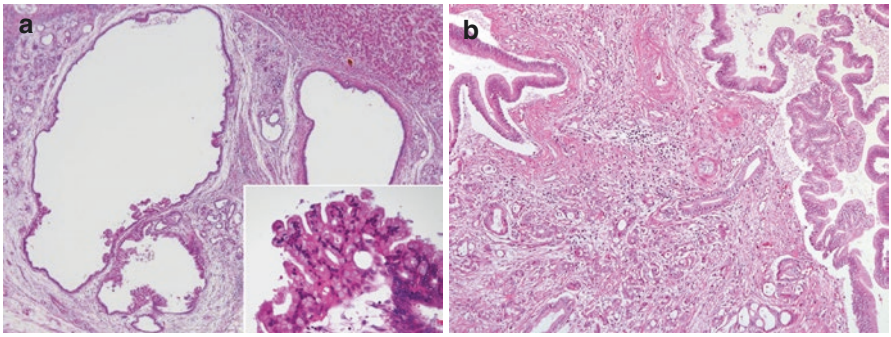


**Fig. 14.4** Cystic and micropapillary lesions of peribiliary glands. (**a**) Cystic change of the peribiliary glands accompanied by micropapillary epithelial proliferation (*inset*). (**b**) Cystic and micropapillary lesion surrounded by invasive adenocarcinoma. Hematoxylin and eosin staining

change. Among 938 cases, cystic and micropapillary lesions of the peribiliary glands were observed in 9 cases (1%).

### *14.5.2 Underlying Hepatobiliary Disorders*

Among nine cases having cystic and micropapillary lesions of the peribiliary glands, four cases were associated with liver cirrhosis due to alcohol (one case) and of unknown etiology (three cases). Submassive hepatic necrosis, intrahepatic cholestasis, or pancreatic cancer was noted in one case, respectively. Due to the limited number of cases, it was unclear whether this could be regarded as a particular underlying condition for cystic and micropapillary lesions. It was also observed in normal liver in one case. In addition, one case was accompanied by cholangiocarcinoma at the hepatic hilum (Fig. 14.4b).

### *14.5.3 Histopathological Characteristics*

The mean size of cystic glands was 3.9 mm in diameter, in which the maximum size was 6.0 mm. In each case with cystic and micropapillary lesions, they were usually observed as multiple foci, and the peribiliary glands with microcystic change less than 2 mm in diameter were also invariably admixed in the same case.

Histologically, cystic and micropapillary lesions were characterized by a micropapillary epithelial configuration with either no or few fibrovascular cores (Fig. 14.4a). The stroma was with or without significant inflammation. Micropapillary epithelial change was observed in the peribiliary glands of more than 2 mm in diameter in two of nine cases, and in other cases, the epithelial change was confined to dilated peribiliary glands of less than 2 mm in diameter. In two cases, there was extension of the lesion into the large bile ducts adjacent to the lesion.

The papillae of cystic and micropapillary lesions were line by columnar epithelial cells with abundant apical mucin, which was more clearly recognizable in the sections stained with Alcian blue. The abundant mucin expression was similar to that seen in mucinous acini of normal extramural peribiliary glands, raising the possibility that cystic and micropapillary lesions might arise from mucinous acini of the peribiliary glands.

The cytologic atypia of cystic and micropapillary lesions was usually mild, corresponding to low-grade dysplasia. In one case of a cystic and micropapillary lesion associated with surrounding cholangiocarcinoma, a cystic and micropapillary lesion corresponding to high-grade dysplasia was observed (Fig. 14.4b). Similar to this case, there has been a report showing the occurrence of cystic micropapillary neoplasm of the peribiliary glands with concomitant perihilar cholangiocarcinoma [29].

### *14.5.4 Immunohistochemical Findings*

In cystic and micropapillary lesions, cytoplasmic expression of MUC5AC and cytoplasmic and nuclear expression of S100P were frequently observed. Approximately half of the cases with cystic and micropapillary lesions showed nuclear expression of cyclin D1 and membranous and cytoplasmic expression of CEA but negative or faint expression of MUC1. MUC2 and p53 were totally negative in the lesions. The Ki-67 labeling index was increased in cystic and micropapillary lesions with the mean value of 10%.

During the multistep cholangiocarcinogenesis, it has been reported that the immunohistochemical expression of MUC5AC, cyclin D1, and S100P is increased in preinvasive lesions such as biliary intraepithelial neoplasia and intraductal papillary neoplasm of the bile duct (IPNB) as well as cholangiocarcinoma [12, 23]. Since epithelial atypia of cystic and micropapillary lesions was usually mild, some cases of cystic and micropapillary lesions might represent multifocal hyperplasia of the peribiliary glands. However, the results of histological analysis suggest that cystic and micropapillary lesions may have neoplastic features, and the elevation of Ki-67 labeling index of cystic and micropapillary lesions also supports this consideration.

### *14.5.5 Resemblance to Branch-Type IPMN of the Pancreas*

Branch-type intraductal papillary mucinous neoplasm (IPMN) of the pancreas is well characterized. It usually shows gastric type, and abundant cytoplasmic mucin can be demonstrated. Generally, gastric-type IPMN proves to have only low- or intermediate-grade dysplasia. Gastric-type IPMN immunohistochemically labels for MUC5AC, but not MUC1 and MUC2. In addition, the expression of cyclin D1 increases in frequency from IPMN with low-grade dysplasia to those with high-grade dysplasia [1]. The expression of S100P has been detected in nearly all cases of IPMN, but normal pancreatic ductal epithelium lacks its expression [20]. Although cystic and micropapillary lesions of the peribiliary glands are usually less than 5 mm in diameter, other histopathological characteristics of cystic and micropapillary lesions seem to resemble to those of branch-type IPMN.

## 14.6 IPNB Involving Peribiliary Glands

IPNB is a concept originally described as a preinvasive neoplasm that corresponds to a biliary counterpart of IPMN of the pancreas [18]. Most of the previously reported cases of IPNB were located in the extrahepatic and large intrahepatic bile

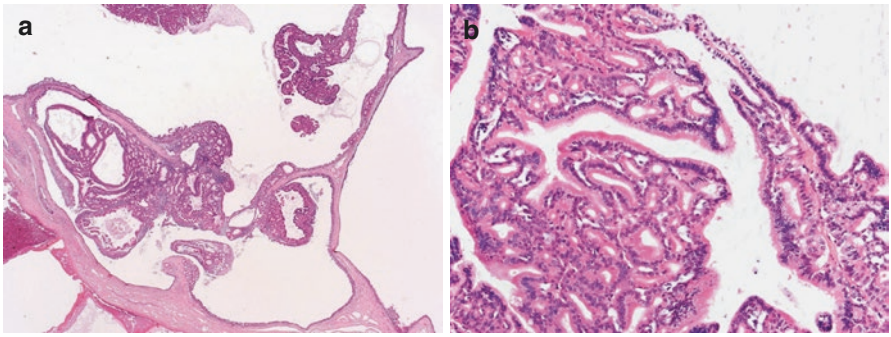


**Fig. 14.5** Cystic and papillary neoplasm of peribiliary glands. (**a)** Epithelial proliferation in the peribiliary glands with cystic change. (**b)** Gastric-type papillary and glandular components within the lesion. Hematoxylin and eosin staining

ducts. Recently, there have been several reports of IPNB that might have arisen from the peribiliary glands [10]. It has been proposed that IPNB involving the peribiliary glands can be called as branch-type IPNB based on the similarity to branch-type IPMN of the pancreas [19].

For example, a case in which cystic and papillary neoplastic lesions only involved in the peribiliary glands has been reported, and this case might correspond to branch-type IPNB [14] (Fig. 14.5). In another case, IPNB showed diverticular dilatation, and neoplastic changes involving the peribiliary glands were found at the tip of the diverticular dilatation [13]. In the latter case, papillary neoplastic changes were also continuously found on the luminal surface of the neighboring bile ducts, and it seems possible that the case corresponds to the combined branch- and main-duct type IPNB. In addition, intraductal tubulopapillary neoplasm which might arise from peribiliary cyst has been reported [31].

There is a possibility that cystic and micropapillary lesions of the peribiliary glands may represent precursors of branch-type IPNB, although the characteristics of branch-type IPNB are largely unknown due to rare incidence of the neoplasm.

## 14.7 Conclusions

In this chapter, histopathological features of the peribiliary glands associated with cystic and micropapillary epithelial changes have been described. They may have neoplastic features, and histological pictures are similar to those of branch-type IPMN of the pancreas other than the microscopic size of ductal diameter. They may be precursors of biliary epithelial neoplasms involving IPNB as well as cholangiocarcinoma. Although the clinical significance of the lesion remains uncharted, the recognition of the lesion seems to be important in biliary tract pathology when the biology and oncogenesis of biliary epithelial neoplasms are considered.

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# Chapter 15
# Intraepithelial Neoplasia of Bile Ducts in Nodular Sclerosing Cholangiocarcinoma: Heterogeneous Categories

**Yasuni Nakanuma, Tsuneyoshi Uchida, and Yoshifumi Ohnishi**

**Abstract** The most common gross type of cholangiocarcinoma (CCA) of intrahepatic large bile duct and perihilar and distal bile ducts is the nodular-sclerosing type (NS-CCA). Biliary intraepithelial neoplasia (BilIN) is the term to describe flat or micropapillary or papillotubular dysplastic epithelium in the bile duct and is proposed as a preceding lesion of NS-CCA, particularly those associated hepatolithiasis. Its three-grade classification (BilIN-1, -2, and -3) may reflect multistep carcinogenesis of NS-CCA, and BilIN-3 is regarded as carcinoma in situ. Other intraepithelial growth forms of biliary neoplasia are also not infrequently found in the bile ducts around invasive NS-CCA: intraepithelial invasion of periductal invasive carcinoma through the basement membrane of the bile duct (cancerization). Cancerization in the biliary epithelial layer is a variably differentiated adenocarcinoma and presents flat, micropapillary, and papillotubular patterns. Furthermore, in NS-CCA without preceding chronic biliary diseases, intraepithelial neoplasms are also frequently found in the bile ducts; this lesion may be composed of preinvasive neoplasm (corresponding to BilINs) and cancerization from invasive NS-CCA. In conclusion, the intraepithelial neoplasms of the bile ducts in NS-CCAs could be heterogeneous, and the recognition of these subcategories of intraepithelial

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neoplasms is important for the evaluation of the development of CCAs, and it is also practically important whether surgical margin of bile duct with intraepithelial neoplasm is BilINs or cancerization.

**Keywords** Biliary tree • Cholangiocarcinoma • Cancerization • Intraepithelial neoplasm • Invasion

## Abbreviations

| | |
|---|---|
| BilIN | Biliary intraepithelial neoplasia |
| CCA | Cholangiocarcinoma |
| CEA | Carcinoembryonic antigen |
| IENB | Intraepithelial neoplasm of bile duct |
| NS-CCA | Nodular-sclerosing cholangiocarcinoma |
| PanIN | Pancreatic intraepithelial neoplasm |
| PDAC | Pancreatic duct adenocarcinoma |
| PSC | Primary sclerosing cholangitis |

## 15.1 Introduction

Currently, cholangiocarcinomas (CCAs) are classifiable into intrahepatic CCA, perihilar CCA, and distal CCA according to its anatomical location [1]. Intrahepatic CCA is further divided into peripheral intrahepatic CCA and large bile duct intrahepatic CCA, and the latter is grossly classifiable into mass-forming, periductal-spreading, and intraductal growth type. Perihilar and distal CCAs are grossly divided into flat/nodular growth type, intraductal papillary type, and others [1]. Interestingly, periductal-spreading type of large bile duct intrahepatic CCA and flat/nodular growth type of perihilar and distal CCA share many features such as nodular/flat sclerosis of bile ducts associated with infiltration of carcinoma cells in the ductal wall and periductal tissues, so these types are now collectively called as nodular-sclerosing (NS) CCA in this review. NS-CCA is the most common type of CCA affecting intrahepatic large bile ducts and perihilar and distal bile ducts ("conventional CCA" [1] ).

While NS-CCA is a highly malignant neoplasm characterized by early invasion and metastasis, there have been several reports on the intraepithelial neoplastic lesion or field on the bile duct mucosa around the main tumor(s) of conventional CCA [2–5]. Recently, biliary intraepithelial neoplasia (BilIN) has been proposed as a preinvasive intraepithelial lesion of the large intrahepatic and extrahepatic bile ducts and also peribiliary glands and the gallbladder of NS-CCA by WHO classification of tumors of the digestive system [6, 7]. Sato et al. reported that in addition to BilINs, intraepithelial back invasion of periductal invasive carcinoma through the basement membrane of the bile duct (cancerization) was also recognizable

as an intraepithelial growth of biliary neoplasm in the bile ducts of NS-CCA associated with hepatolithiasis [5, 8], suggesting that the intraepithelial neoplastic lesions could be heterogeneous and at a chimera state in the bile ducts in such NS-CCA.

Sakamoto et al. firstly reported that mucosal (intraepithelial) extension of carcinoma was seen not infrequently in hilar CCA without chronic biliary diseases [2]. Subsequent studies showed that intraepithelial extension of carcinoma without subepithelial infiltration of carcinoma at the surgical margin has no prognostic significance [3, 4]. However, these studies did not refer to preinvasive intraepithelial neoplasm and cancerization (backward intraepithelial invasion) in the evaluation of intraepithelial extension of carcinoma.

S100P is a member of the S100 family of calcium-binding proteins, and S100P expression at high levels has been found in a variety of different types of tumors. There have been several reports of its aberrant expression in pancreatic duct adenocarcinoma (PDAC) and also CCA and also their early neoplastic and noninvasive lesions such as pancreatic intraepithelial neoplasm (PanIN) and BilIN in hepatolithiasis associated with NS-CCA [5, 8–10], and S100P is used for detection of neoplastic epithelial lesions in the biliary tree [5, 8–10], while strong S100P was not expressed in normal bile ducts and only focally and weakly or heterogeneously expressed in the nonneoplastic bile ducts in noncancerous biliary diseases.

Herein, we review the pathologies and pathological significance of BilIN and cancerization in the bile ducts of NS-CCA arising in hepatolithiasis [8] and then characterize intraepithelial neoplastic lesions in the bile ducts of NS-CCA without preceding chronic biliary diseases with a help of immunostaining of S100P [5].

## 15.2 Intraepithelial Neoplasms in NS-CCA Associated with Chronic Biliary Diseases

In long-standing biliary diseases such as hepatolithiasis and primary sclerosing cholangitis (PSC), CCA is known to develop eventually in approximately 5% of the patients with hepatolithiasis and 10% of PSC patients [1, 8]. The stone-containing bile duct presents proliferation of biliary lining epithelia and peribiliary glands in addition to ductal and periductal fibrosis and inflammatory cell infiltration (chronic proliferative cholangitis) [8, 9]. NS-CCA arising in hepatolithiasis usually proliferates as flat or micropapillary or papillotubular carcinoma on the luminal surface of the affected bile ducts as well as invades the ductal wall as tubular adenocarcinoma of variable differentiation with desmoplasia. In addition, NS-CCA with hepatolithiasis is frequently associated with atypical lining epithelia (recently called as BilIN, see below) in the biliary mucosa of chronic proliferative cholangitis around or in the vicinity of CCA, and not only are carcinoma cells of CCA positive for carcinoembryonic antigen (CEA) and other tumor markers such as aberrant expression of EZH2 and cyclin D1 [10], BilINs also show positive stain for them frequently, suggesting that such atypical biliary lesions could be a kind of premalignant or preinvasive lesions.

### *15.2.1 BilIN*

BilIN was coined to describe atypical intraepithelial neoplastic lesions in the bile duct and is used instead of several traditional terms such as atypical biliary epithelia or biliary epithelial dysplasia [1, 6, 7]. So far, the histopathological and molecular characteristics of BilINs have been mainly examined and established by using chronic biliary diseases, particularly hepatolithiasis with and without CCAs [9], so the application of BilIN system to the bile ducts and gallbladder with or without NS-CCA without preceding chronic biliary diseases should be careful and needs further studies. BilINs are histologically characterized by flat, pseudopapillary, micropapillary and papillotubular lesions, usually less than 3 mm in their height. BilINs are macroscopically and radiologically unidentifiable, specificially.

The grading is based on the histological appearance of H&E-stained sections and is determined according to the degree of atypia of intraepithelial lesion such as loss of cellular/nuclear polarity, increased nucleus-to-cytoplasmic ratio, and nuclear hyperchromasia. The three-tiered classification system is applied: BilIN-1 (low-grade lesion), BilIN-2 (intermediate-grade lesion), and BilIN-3 (high-grade lesion) (Fig. 15.1). BilIN-3 is a preinvasive lesion (carcinoma in situ) of NS-CCA [6, 7] and

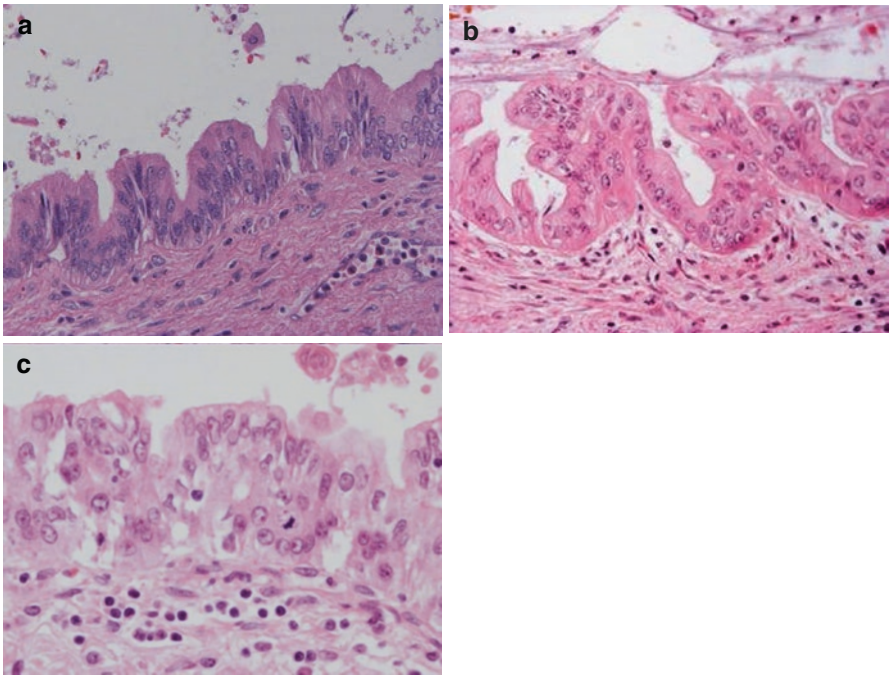


**Fig. 15.1** Biliary intraepithelial neoplasia (BilIN)-1,2,3 in hepatolithiasis. (**a**) BilIN-1, H&E, x300 (original magnification). (**b**) BilIN-2, H&E, x300 (original magnification). (**c**) BilIN-3, H&E, x300 (original magnification)

is usually seen in cases of invasive NS-CCA [8, 9]. For the distinction between BilIN-1 and BilIN-2, piling up of the nuclei from the basal lamina up to the apical surface of the bile duct is a useful morphological feature of BilIN-2, while diffuse disturbance of cellular/nuclear polarity favors BilIN-3. Recent experiences suggest the two-tiered system in which BilIN-1 and BilIIN-2 are grouped as low-intermediate grade (BilIN-1/2) and BilIN-3 as high grade Multiple carcinogenetic processes such as oxidative stress, toxic bile acids, and cellular senescence associated with activation of cholangiocytes in chronic proliferative cholangitis in hepatolithiasis are possibly responsible for the multistep development of preinvasive neoplastic lesions (BilINs) and eventual progression of invasive NS-CCA [10, 11].

As for differentiation from BilIN, the biliary tract is often affected by inflammatory conditions; strong and diffuse expression of S100P in an epithelial field or lesion favors BilINs than reactive changes of biliary epithelial cells.

### *15.2.2 Cancerization*

In addition to BilIN, another form of intraepithelial neoplasm, cancerization, is known to develop in the bile ducts of NS-CCA [5, 8, 9]. Sato et al. reported that in the bile ducts of CCA associated with hepatolithiasis, "intraepithelial spread of carcinoma (IES)" reflecting cancerization was found in addition to BilIN-3 [8,9]. That is, IES is continuously adjacent to the main tumor of NS-CCA, and these lesions reflect cancerization characterized by a direct intraepithelial invasion of periductal invasive carcinoma through the basement membrane of the nonneoplastic bile ducts. Such cancerization is also frequent in entrapped nonneoplastic bile ducts within the main tumor (Fig. 15.2). Cancerization is usually composed of more atypical or bizarre carcinoma cells in comparison with BilIN-3. Phenotypes of carcinoma cells such as excessive expression of S100P and p53 are found in invasive tubular adenocarcinoma and also in cancerized intraepithelial neoplasm in the bile duct (Fig. 15.2c, b), while p53 expression is infrequent in BilINs. Front formation between cancerized intraepithelial carcinoma cells and nonneoplastic lining biliary epithelia is evident here in the bile duct mucosa. Cancerization is usually associated with subepithelial spread of invasive carcinoma within and outside the ductal wall (submucosal invasive spread).

By considering the differential findings and characteristics of these two lesions, Sato et al. reported that BilIN-3 and LSIN were observed in 17 cases (94%) and seven cases (39%), respectively, in 18 hepatolithiasis cases of invasive CCA. BilINs may remain and are possibly intermingled or admixed in cancerization of invasive NS-CCAs in hepatolithiasis [8]. Thus, intraepithelial neoplasm in the biliary tract of hepatolithiasis with NS-CCA could be at a chimera state of BilINs and cancerization.

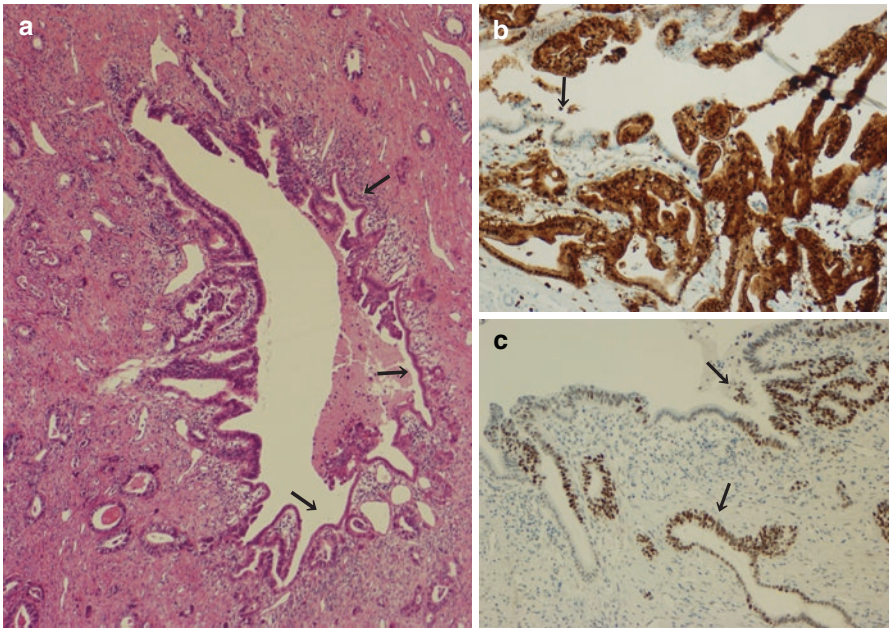


**Fig. 15.2** Cancerization (intraepithelial invasion of periductal invasive carcinoma through the basement membrane of the bile duct). (**a**) Periductal carcinoma cells are invading into the lining epithelia of the bile duct and push aside the nonneoplastic biliary epithelial cells. Nonneoplastic biliary epithelium (→) in the bile duct remains here and there. H&E, x300 (original magnification). (**b**) Immunostaining of S100P. Cancerized epithelia and periductal infiltrating carcinoma are strongly positive, while the remaining nonneoplastic biliary epithelia (→) are negative. x150 (original magnification). (**c**): Immunostaining of p53. Cancerized carcinoma cells and periductal infiltrating carcinoma cells (→) are strongly and diffusely positive for p53, while the remaining nonneoplastic biliary epithelia are negative. x150 (original magnification)

## 15.3 Intraepithelial Neoplasm of Bile Ducts in Invasive NS-CCA Without Chronic Biliary Diseases

So far, there have been several reports about intraepithelial neoplasm such as "mucosal extension of carcinoma" or "intraepithelial spread of carcinoma" in the bile ducts in invasive NS-CCA without chronic biliary diseases [2–5]. However, in these reports, the concept of preinvasive neoplastic lesions or cancerization was not taken into consideration. Our recent study showed that several categories of intraepithelial neoplasm such as preinvasive neoplasm and cancerization were also recognizable in the bile ducts of NS-CCA without chronic biliary diseases [5].

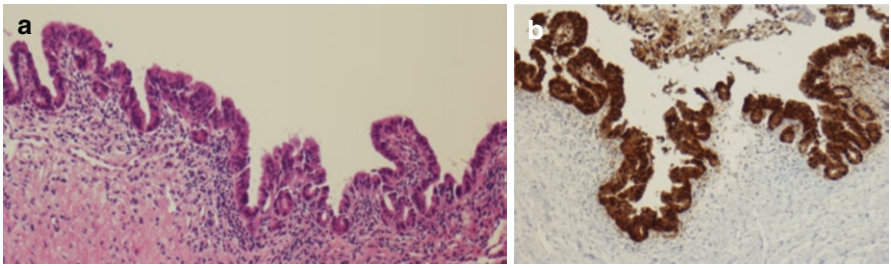


**Fig. 15.3** Group A lesion of intraepithelial neoplasm of bile duct. (**a)** Intraepithelial neoplasm showing nuclear stratification and mild hyperchromasia. Nuclear polarity is preserved. Glandular formation is found at the basal side of this lesion. Corresponding to BilIN-1/2 (low to intermediate dysplasia). HE, x120 (original magnification). (**b)** Intraepithelial neoplasm showing strong and diffuse staining of S100P. Immunostaining of S100P and hematoxylin, x100 (original magnification)

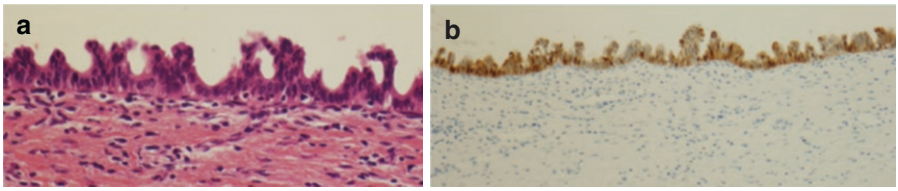


**Fig. 15.4** Group B lesion of intraepithelial neoplasm of bile duct. (**a)** Intraepithelial neoplasm showing nuclear stratification and mild hyperchromasia and pseudopapillary pattern. Nuclear polarity is disturbed. Corresponding to BilIN-3 (high-grade dysplasia or in situ carcinoma). HE, x130 (original magnification). (**b)** Intraepithelial neoplasm showing strong and diffuse staining of S100P. Immunostaining of S100P and hematoxylin, x100 (original magnification)

### *15.3.1 Intraepithelial Neoplasm of Bile Duct (IENB)*

It is possible that the intraepithelial spreads of carcinoma in the bile ducts of NS-CCA are heterogeneous. So, first, we surveyed collectively intraepithelial neoplasm in the proximal and distal bile ducts in invasive NS-CCA as "intraepithelial neoplasm of bile duct (IENB)." IENB was defined histologically as a field of intraepithelial neoplastic biliary epithelial cells composed of variably differentiated, cuboidal to columnar cells on the bile duct and also in the peribiliary glands in the bile ducts of the main tumor(s) of NS-CCA (Figs. 15.3, 15.4, and 15.5). IENB constantly expressed S100P strongly and diffusely in the nuclei and/or cytoplasm of epithelial cells and formed a continuous S100P-positive biliary epithelial lesion or field (Figs.15.3b, 15.4b).

Some of IENBs were associated with subepithelial infiltration of carcinoma in the duct wall while the other not associated. The IENB with subepithelial infiltration

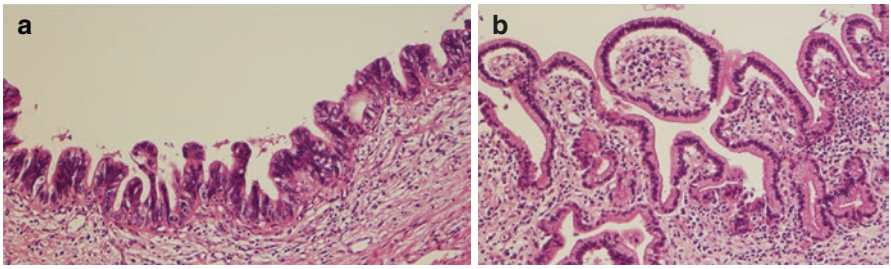


**Fig. 15.5** Group C of intraepithelial neoplasm of bile duct (intraductal spread of cancerization). (**a**) Intraepithelial neoplasm showing nuclear hyperchromasia, anisonucleosis, irregular-shaped nucleus, and pseudopapillary pattern. HE, x130 (original magnification). (**b**) Single-layered atypical cells with hyperchromasia and thickened nuclear membrane. Immunostaining of S100P and hematoxylin, x100 (original magnification)

of carcinoma was usually adjacent or continuous to the main tumors of NS-CCA and could be a component of invasive CCA, so only IENBs without subepithelial infiltration of carcinoma cells were examined. Then, we classified IENB into three types based on their cellular and structural atypia.

### *15.3.2 IENB Can Be Divided into Three Types*

IENB showed flat or waving pattern including pseudostratification, pseudopapillary, micropapillary, or papillotubular of neoplastic epithelial cells. Grossly, this lesion was fine granular or rough or velvety or unremarkable on the biliary mucosa.

IENB showed variable differentiation and could be histologically classified into three categories according to their atypia: group A (neoplastic but not be enough for malignancy) (Fig. 15.3), B (neoplastic and well differentiated enough for high-grade dysplasia or in situ carcinoma) (Fig. 15.4), and C (overtly malignant and variably differentiated) (Fig. 15.5) [5]. IENB was found in more than a half of NS-CCA cases without chronic biliary diseases: Group C was the most frequent followed by group B and then group A in NS-CCA. These three types were frequently coexisted in the same case of NS-CCA.

**Group A and B** The size of neoplastic cells and their nuclei of these groups were comparable or a little large or small to nonneoplastic epithelial cells of large bile ducts, and their nuclei are more or less round or oval and constantly show variable stratification with occasional piling up to the lumen and nuclear hyperchromasia. The groups A and B were frequently associated or admixed with pyloric gland formation at their bases (Fig. 15.3). Group A may show gradual transition to adjacent normal biliary epithelium, while group B shows constantly an abrupt transition to surrounding epithelium. Groups A and B do not share cytological and structural features with carcinoma cells covering or infiltrating the main tumor of invasive CCAs. **Group A** appeared rather homogeneous and their nuclear polarity was not

disturbed (Fig. 15.4). **Group B** showed cellular pleomorphism variably and also showed evident nuclear hyperchromasia and disordered nuclear polarity.

**Group C** This group is obviously malignant, is variably differentiated, and showed micropapillary or pseudopapillary or flat lesion and/or also single layer of overt malignant epithelial cells (Fig. 15.5). This group shows nuclear changes enough for malignancy including thickening of nuclear membrane, irregular shape such as rectangular or square shape, pleomorphism and prominent nucleoli, loss of polarity, and cellular anisocytosis including bizarre cells. Group C is frequently continuous with intraepithelial biliary neoplasm with subepithelial infiltrative carcinoma. Group C is rarely associated with pyloric gland changes at their basis.

As for subtypes, all three groups of IENBs showed strong and diffuse CK 7 and frequent MUC1 expression, thus reflecting a pancreatobiliary subtype [1, 6], though they also showed frequently gastric and intestinal markers such as MUC2, MUC5AC, and MUC6.

## *15.3.3 Clinicopathological Significance*

### 15.3.3.1 IENB Can Be Classified into Preinvasive Lesions and Cancerization

Group C was frequently found in the bile ducts of NS-CCAs and shared cytological features of infiltrating carcinomas in the main tumor of NS-CCAs. In contrast, cytological features of groups A and B were different from those of NS-CCA. Expression of cancer-related markers of group C was lower than but relatively similar to that of invasive CCA, while that of groups A and B was different from invasive CCAs. For example, strong immunostaining of P53 was frequently positive in invasive CCAs, though its positivity was relatively low in group C and infrequent in group B and rare in group A. S100A and reduced expression of E-cadherin in carcinomas are reported to reflect epithelial mesenchymal transition and also increased migration and metastasis of carcinoma cells. S100A was expressed in almost all cases of invasive CCA, and its expression was frequent in group C, while its expression was focal and infrequent in groups B and A. Reduction of E-cadherin expression was common in invasive CCA, while its reduction was frequent in group C, infrequent in group B, and absent in group A. As for Ki67 index, proliferative activities were higher in invasive CCA followed by groups C, B, and A in this decreasing order.

Taken together, it seems plausible that cancerization of periductal infiltrating CCA may be included in group C. Groups A and B were different from infiltrating carcinoma of NS-CCA and could be a preinvasive intraepithelial neoplasm of NS-CCA. Therefore, it seems conceivable that there can be at least two categories of intraepithelial neoplasm in the bile ducts in NS-CCA without preceding chronic biliary disease as in NS-CCA arising in hepatolithiasis. With consideration of histologic features of these lesions in comparison with BilINs, group A may correspond to low- to intermediate-grade dysplasia (BilIN-1,2) and group B to high-grade

dysplasia or carcinoma in situ (BilIN-3). It is of interest that group B was frequently found in the bile ducts around invasive NS-CCA, suggesting that group B may be a frequent preinvasive cancerous lesion eventually followed by invasion of carcinoma. Similar tumorigenetic mechanism(s) or tumor progression as speculated in hepatolithiatic patients with BilINs and NS-CCA may be also operative in these cases showing group B without chronic biliary diseases. Further studies are mandatory to clarify the mechanisms of group B and group A lesions in NS-CCA without "clinically nonapparent" chronic biliary diseases.

#### 15.3.3.2 Significance of Subcategories of IENB

As for the prognostic significance of mucosal extension of carcinoma, the survival for the patients with remnant carcinoma in situ does not differ from that for those without remnant carcinoma [2–4], though there are several reports that a few patients with remnant carcinoma in situ caused local recurrence 7–10 years later in the postoperative phase. These reports raise a possibility that "intraepithelial spread of carcinoma" without subepithelial infiltration of carcinoma in the bile ducts in NS-CCA, particularly remnant carcinoma in situ in the bile ducts, has no or few capacity of invasion, strongly suggesting that remnant carcinoma in situ may be preinvasive lesions such as BilINs and group B in our study [1, 6] and these lesions could take a longer time to recur or invade.

As mentioned above, this "intraepithelial spread of carcinoma" in the bile ducts of NS-CCAs may be composed of at least two categories (preinvasive lesions and cancerization), and it is likely that the former could be frequent in more peripheral parts of bile ducts from NS-CCAs and could become the surgical resection margin at the time of surgical resection of NS-CCAs rather than cancerization. So far, there have been no studies on cancerized epithelial cells and the fate of the bile ducts with cancerization, though it is plausible that cancerization is able to invade and recur, if this lesion remains at the surgical margin after operation. However, it remains unclear whether the patients with surgical margin of cancerized epithelia in the bile ducts without subepithelial infiltration of carcinoma present different prognosis from the patients with preinvasive lesions at the surgical margins. It is also unclear whether the cancerized bile ducts and the bile ducts with preinvasive neoplastic lesions show different pathological and biological behaviors or not.

## 15.4 Similarities to PDAC and Pancreatic Intraepithelial Neoplasm

### *15.4.1 Biliary Diseases with Pancreatic Counterparts*

The biliary tree and pancreas are closely located anatomically and share several developmental processes and physiological functions [12]. Experimental studies using animal models have suggested that the biliary tract holds some potential for

pancreatic differentiation. In humans, peribiliary glands are located around the biliary tract and drain into the bile duct lumen via their own conduits. Interestingly, small amounts of pancreatic exocrine acini are intermingled with these glands, suggesting the possibility that these glands may be abortive pancreatic exocrine acini. Recently, biliary tract stem cells were found in the peribiliary glands and were shown to be able to differentiate into pancreatic cells as well as cholangiocytes. Based on these findings, it seems plausible that the biliary tract has pancreatic features (incomplete pancreas) in addition to a duct system specialized for the drainage of bile secreted by the hepatic parenchyma. In this context, some biliary diseases would have similar pathological features and even biological behaviors as pancreatic diseases ("biliary diseases with pancreatic counterparts") [12].

### *15.4.2 Intraepithelial Neoplasm and Cancerization in the Bile Duct and Pancreas*

Similarities between invasive NS-CCAs and PDAC, and those between BilINs and the precursor lesions of PDAC, particularly PanINs, have been reported [1, 6]. Therefore, it is possible that similar types of intraductal, intraepithelial spreads can also occur in the pancreatic ducts around and remote from the invasive PDACs. In fact, in PDAC, PanINs including high-grade PanIN are variably seen in the surrounding pancreatic ducts, unifocally or multifocally [13]. In addition, infiltrating carcinoma of PDAC is known to invade back into the pancreatic duct lining epithelia and extend along the ductal system (cancerization). Cancerization of the ducts is common and has been reported in as many as 70% of resections for invasive PDAC. This lesion (cancerization) morphologically mimics intraductal neoplasia such as high-grade PanIN [13]. So, intraepithelial lesions in the pancreatic ducts could be heterogeneous and at a chimera state of preinvasive lesions and cancerization as in the bile ducts of NS-CCA.

## 15.5 Conclusion

In invasive NS-CCA with or without preceding chronic biliary diseases, intraepithelial neoplasms are frequently found in the bile ducts; this lesion may be composed of at least two categories: preinvasive neoplasm (BilINs including "carcinoma in situ") and cancerization from invasive NS-CCA. NS-CCAs are intractable malignant neoplasms arising from the intrahepatic and extrahepatic bile ducts, and surgical resection is the only curative therapy of NS-CCAs. However, CCAs are usually detected and diagnosed at an advanced stage, and most CCAs have a poor prognosis even after surgical resection. So, early detection of NS-CCA at a preinvasive stage such as BilINs is the only hope of curing these highly malignant diseases; further studies on and early detection of preinvasive intraepithelial neoplasm are mandatory

to overcome NS-CCAs. In addition, the follow-up and comparative studies of the patients with surgical margin showing intraepithelial neoplasm based on BilINs or cancerization are mandatory to evaluate the prognosis of the patients with the so-called positive surgical margin without stromal invasion at operation.

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# Chapter 16
# Intraductal Papillary Cystic Neoplasm of the Gallbladder and the Ampulla of Vater

**Nobuyuki Ohike, Volkan Adsay**

**Abstract** Intraluminal mass-forming preinvasive neoplasms occurring within the gallbladder and the ampulla of Vater are highly analogous to pancreatic or biliary intraductal papillary and tubular neoplasms, as evidenced by their papillary and/or tubular growth, variable cell lineage, and spectrum of dysplastic change (adenoma-carcinoma sequence), often with significant overlap. A uniform terminology of intracholecystic papillary-tubular neoplasm (ICPN) and intra-ampullary papillary-tubular neoplasm (IAPN) has therefore been proposed for the systematic analysis of these neoplasms. ICPN and IAPN are biologically indolent; noninvasive examples show an excellent prognosis, whereas those with invasion exhibit a malignant but nevertheless significantly better prognosis than ordinary invasive carcinomas unaccompanied by ICPN or IAPN.

**Keywords** Intracholecystic papillary-tubular neoplasm (ICPN) • Intra-ampullary papillary-tubular neoplasm (IAPN) • Pathology • Preinvasive neoplasm • Mass-forming invasive carcinoma

## Abbreviations

| | |
|---|---|
| ICPN | Intracholecystic papillary-tubular neoplasm |
| IAPN | Intra-ampullary papillary-tubular neoplasm |
| IPMN | Intraductal papillary mucinous neoplasm |
| IPNB | Intraductal papillary neoplasm of the biliary tract |
| BilIN | Biliary intraepithelial neoplasia |

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## 16.1 Introduction

Two types of precancerous or preinvasive lesions precede invasive adenocarcinomas of the gallbladder and the ampulla of Vater: the flat and non-mass-forming type and the papillary or polypoid mass-forming type.

The flat and non-mass-forming type is a microscopically defined flat or at most micropapillary lesion characterized by columnar epithelial cells with multilayering of more or less abnormal nuclei and has been named "biliary intraepithelial neoplasia (BilIN)" in the gallbladder or "flat intraepithelial neoplasia/dysplasia" in the ampulla of Vater, respectively [1, 2].

In contrast, the papillary or polypoid mass-forming type is macroscopically visible, preinvasive lesions forming exophytic mucosal tumors composed of papillary and/or tubular growth of dysplastic epithelial cells. If its diameter exceeds 1 cm, clinically, the lesion may be detectable or symptomatic, and histologically, high-grade dysplastic changes are commonly observed. Such lesions have received many different names, including "intestinal adenoma," "villous adenoma," "tubulopapillary adenoma," "biliary adenoma," "pyloric gland adenoma," "transitional adenoma," "papillary neoplasm," "papillary carcinoma," "intracystic papillary neoplasm," and "noninvasive pancreatobiliary papillary neoplasm."

These names refer to either the growth pattern, the histological phenotype, or the degree of neoplastic change. The WHO 2010 classification compiled all of these previous terms into two categories: "adenoma" vs. "intracystic papillary neoplasm" in the gallbladder and "adenoma" vs. "noninvasive pancreatobiliary papillary neoplasm" in the ampulla of Vater [1, 2].

However, no criteria have yet been established for qualifying a lesion as papillary neoplasm rather than adenoma based on papilla formation nor have any criteria described the degree of high-grade dysplasia allowable in the "adenoma" category. To circumvent these difficulties and as an analogy to the pancreas and the bile duct, the terms "intracholecystic papillary-tubular neoplasms (ICPNs)" and "intraampullary papillary-tubular neoplasm (IAPN)" were recently proposed for all mass-forming precancerous or preinvasive lesions of the gallbladder and ampulla of Vater, respectively [3, 4].

Adopting such uniform terminology may be helpful in several respects, by improving the consistency and reproducibility of the diagnosis even among non-expert pathologists, reducing the confusion in communication between pathologists and clinicians, facilitating the conduct of comparative studies among intraductal papillary neoplasms of the pancreatobiliary system, and reducing the rate of inappropriate treatment due to an alternative diagnosis of "adenoma" or "carcinoma."

Following these proposals, the clinicopathological features about ICPN and IAPN are described.

## 16.2 Overview

ICPN and IAPN are characterized by intraluminal papillary and tubular growth within the gallbladder and ampulla of Vater, respectively (Fig. 16.1), occasionally with excess mucus production. They are rare, but they are clinically detectable, and their rates of detection are increasing with the widespread use of and advances in abdominal imaging and endoscopy methods.

ICPN and IAPN can be noninvasive or associated with invasive carcinoma, usually of the tubular type, although other subtypes (e.g., mucinous) may be encountered. They usually have a favorable prognosis if they do not invade deeply. Noninvasive ICPN/IAPN lesions are cured by surgical resection. Follow-up, however, is needed for early detection of recurrence due to metachronous multifocality.

Similar to intraductal papillary mucinous neoplasm (IPMN) of the pancreas [5] and intraductal papillary neoplasm of the biliary tract (IPNB) [6], ICPN and IAPN are typically characterized by papillary growth with branching fibrovascular stalks lined by atypical cuboidal or columnar cells, showing different degrees of histological

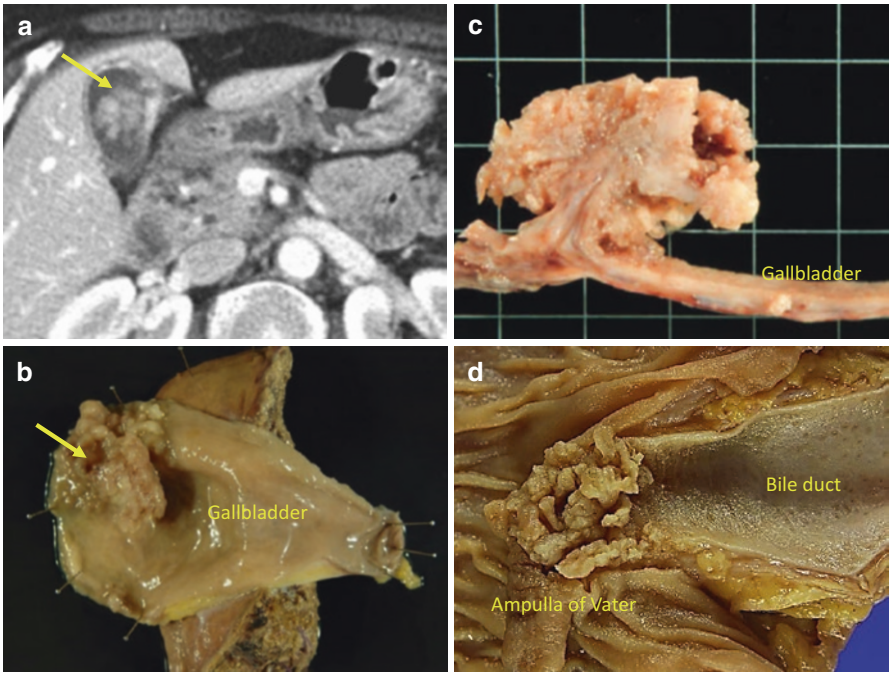


**Fig. 16.1** CT scan and macroscopic features of ICPN (**a–c**) and IAPN (**d**) showing an intraluminal papillary or polypoid mass (**a**, **b**; same case)

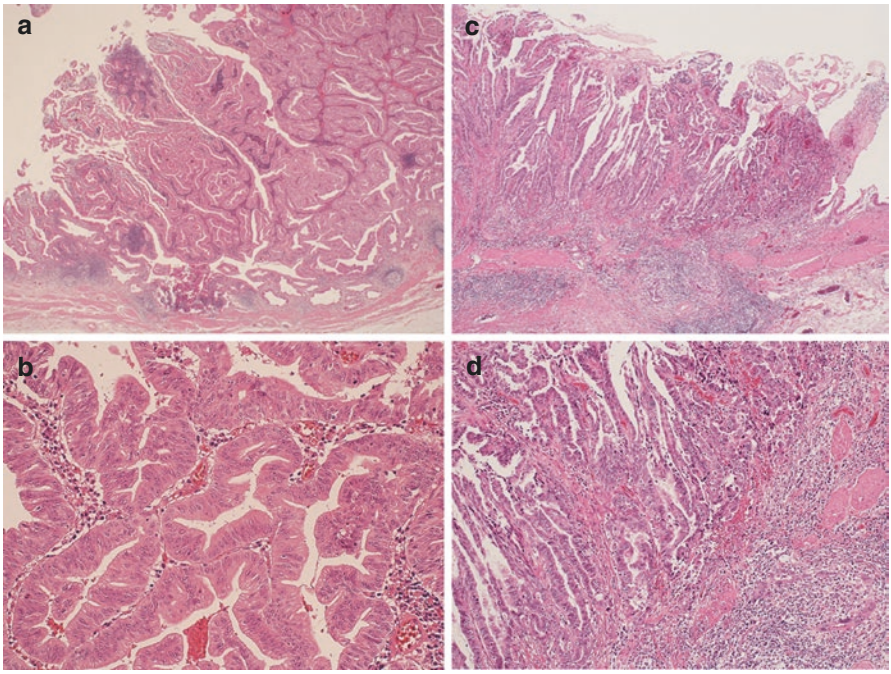


**Fig. 16.2** Microscopic features of ICPN (**a, b**) showing noninvasive papillary growth and invasive papillary "mass-forming" adenocarcinoma of the gallbladder (**c, d**)

dysplasia between low-grade lesions (usually corresponding to adenoma or low-grade carcinoma) and high-grade lesions (usually corresponding to low- or high-grade carcinoma). Of note, these neoplasms contain low-grade dysplastic areas, either focally or substantially, unlike ordinary "invasive papillary adenocarcinomas" or "mass-forming invasive adenocarcinomas," which consist of only extensive distribution of high-grade dysplastic glands and often contain cribriform structure, necrosis, and even invasion within the exophytic mass (Fig. 16.2). In addition, ICPN and IAPN histologically exhibit variable cell lineage, such as pancreatobiliary, intestinal, oncocytic, or gastric phenotype (Fig. 16.3).

**Pancreatobiliary (biliary) type** is characterized by MUC1-positive cuboidal or low-columnar epithelial cells with oval or pencillate nuclei and scanty mucinous, pinkish cytoplasm, resembling the proper epithelium of the pancreatobiliary tract. They show a complex branching and papillary architecture, forming high-grade arborizing papillae (Fig. 16.3a).

**Intestinal type** consists of tubular type and villous type; tubular type is characterized by MUC2-/CDX2-positive tall-columnar epithelial cells with spindle-shaped, pseudostratified nuclei and acidophilic or amphophilic cytoplasm, forming tubular or tubulopapillary structures that resemble colonic tubular adenoma. The presence of goblet cells and Paneth cells is also considered indicative of intestinal lineage (Fig. 16.3b). Villous type is characterized by MUC2-/CDX2- and

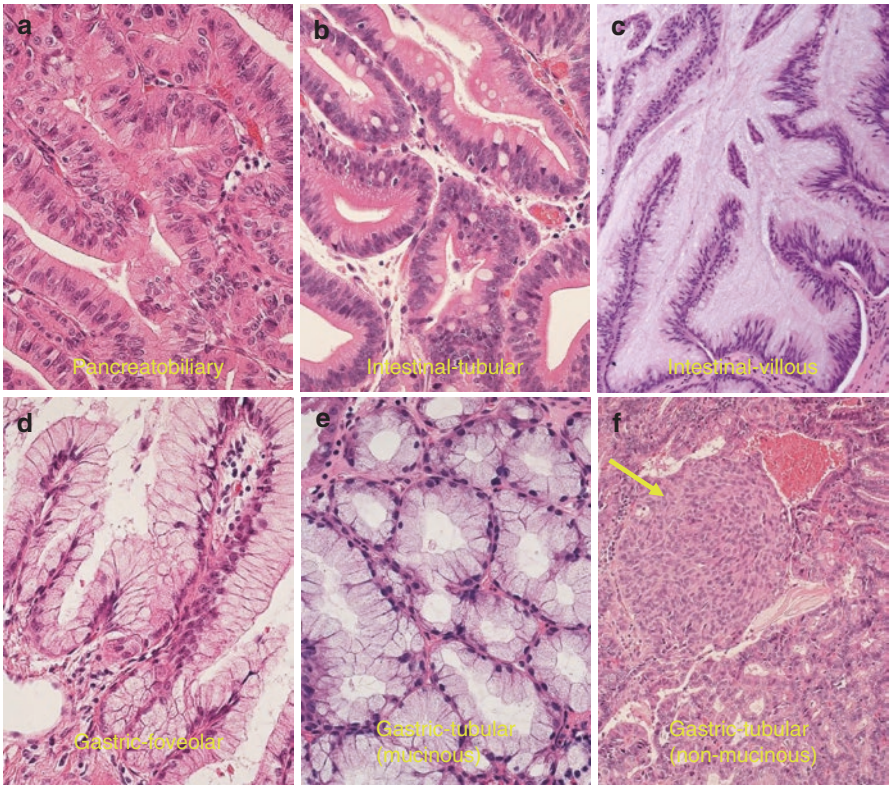


**Fig. 16.3** Microscopic features of variable cell lineage of ICPN or IAPN. Non-mucinous gastric-tubular type (f) shows squamous morular formation (*arrow*)

MUC5AC-positive tall-columnar epithelial cells with spindle-shaped, pseudostratified nuclei and dark mucinous cytoplasm, forming villous structures that resemble colonic villous adenoma (Fig. 16.3c).

**Gastric type** consists of MUC5AC-positive tall-columnar epithelium with clear mucinous cytoplasm, forming papillary structures that resemble gastric-foveolar epithelium (Fig. 16.3d), and MUC6-positive small tubular epithelium similar to the gastric-pyloric glands (Fig. 16.3e).

**Oncocytic type** is characterized by eosinophilic columnar epithelial cells (often MUC6 and HepPar1 positive), with interspersed intraepithelial lumina and goblet cells, forming arborizing papillae and cribriform structures.

If more than one of these characteristics are present simultaneously, the most prominent cell type is used, taking into account predominance and level of atypia. A considerable number of ICPN/IAPN show an overlap in the expression of markers, indicating that they are probably of hybrid nature (Fig. 16.4). In contrast to pancreatic IPMN, the prognostic relevance of the histological subtypes has not yet been established for either ICPN or IAPN.

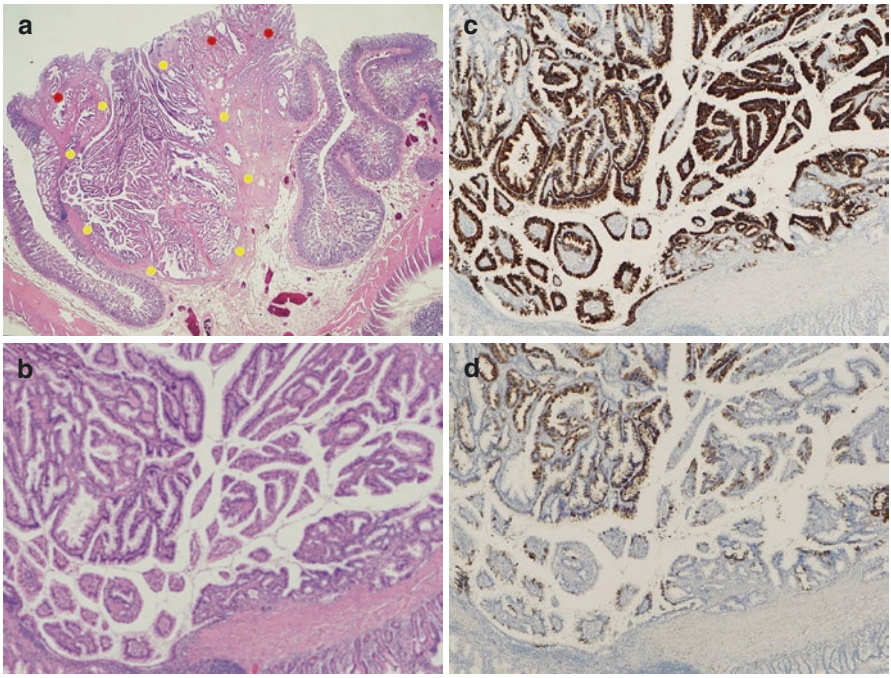


**Fig. 16.4** Microscopic (**a, b**) and immunohistochemical (**c, d**) features of IAPN showing MUC5AC-positive gastric-foveolar and MUC2-positive intestinal-villous phenotypes. *Yellow dot* marks indicate IAPN lesion and red dot marks indicate distended glands (**a**)

**Table 16.1** Mucus and genetic characters according to cell lineages

| Cell lineage | Intestinal-villous | Gastric (foveolar, pyloric) | Pancreatobiliary intestinal-tubular Oncocytic |
|---|---|---|---|
| Excess mucus production | ++ | + | +/− |
| Coexistence of MUC5AC positive | ++ | ++ | +/− |
| Genetic events | KRAS<br>GNAS | KRAS | Unknown |
| | "classical IPMN" | | |

Most cases of ICPN and IAPN belong to the third group

The genetic profiles for these preinvasive lesions are limited. Unlike pancreatic IPMN, ICPN/IAPN may show a low prevalence of KRAS and GNAS mutations in association with a low frequency of the intestinal-villous type and gastric-foveolar type (Table 16.1) [7].

## *16.2.1 ICPN*

### 16.2.1.1 Overview

Recently, Adsay et al. proposed a unified terminology of "intracholecystic papillary-tubular neoplasms (ICPNs)" for all exophytic adenomatous and papillary preinvasive neoplasms of the gallbladder that are ≥ 1.0 cm, regardless of the phenotype of the tumor cells [3]. By definition, ICPNs embrace all subtypes of adenomas and intracystic papillary neoplasms in the WHO 2010 classification [1]. ICPNs occur predominantly in females, with a mean age of 61 years. Gallstones are identified in only 20% of cases. ICPNs are often asymptomatic and occasionally occur in association with Peutz-Jeghers syndrome or Gardner syndrome. Half of cases have an associated invasive carcinoma. Approximately 6% of gallbladder adenocarcinomas arise in association with ICPNs [3].

### 16.2.1.2 Gross Features

ICPNs present as exophytic, well-demarcated polypoid or papillary masses localized within the gallbladder (Fig. 16.1a–c). Most are pedunculated or semipedunculated and solitary but rarely sessile and multinodular. Pedunculated polypoid masses detach readily from the mucosal surface.

### 16.2.1.3 Microscopic Features

ICPNs usually show a mixture of papillary and tubular areas; predominantly, papillary type is the most common (43%), followed by tubulopapillary and tubular. ICPNs also show mixed cellular lineages, but predominantly, pancreatobiliary type is the most common (50%) (Fig. 16.2a, b), followed by gastric-foveolar type and gastric-tubular type. Intestinal type and oncocytic type are rare [3, 7].

Papillary cases tend to be of the pancreatobiliary type, while the gastric phenotype is mostly associated with tubular cases. High-grade dysplasia is more common in papillary and tubulopapillary cases than in tubular ones. In addition, ICPN with an associated invasive carcinoma is more often of the papillary or tubulopapillary type than of the tubular type. The extent of high-grade dysplasia and cell-type (pancreatobiliary or foveolar) and papilla formation are significant factors associated with invasion [3].

Gastric-tubular-type ICPNs (MUC6 positive) are divided into mucinous and non-mucinous types. The mucinous form usually presents as a smaller pedunculated polypoid mass and is similar to pyloric gland adenoma (Fig. 16.3e), while the

non-mucinous types are often larger, complex, pedunculated, multinodular, intraluminal tumors and characterized by complex tubular growth with strong MUC6 expression (indicating pyloric gland differentiation and/or biliary ductular differentiation) and sometimes with scattered squamous morular formation showing nuclear beta-catenin positivity (Fig. 16.3f).

#### 16.2.1.4 Differential Diagnosis

Nonneoplastic polyps, mass-forming invasive carcinoma, and metastatic lesions, which may protrude into the lumen of the gallbladder, must be excluded from the category of ICPN.

1. Nonneoplastic polyps/hyperplastic lesions

   Included in this group are cholesterol polyp, adenomyomatosis (adenomyoma, fibromyoglandular polyp), hyperplastic polyp of the proper epithelium, hyperplastic polyp of the metaplastic epithelium (pyloric gland metaplasia), and fibroepithelial polyp, which are solitary or multiple, pedunculated or sessile, accompanied by epithelial hyperplasia, and usually not larger than 10 mm in size.

2. Invasive papillary, mass-forming adenocarcinoma

   Invasive gallbladder carcinoma is a highly lethal disease and usually shows infiltrative growth with diffuse thickening and induration of the gallbladder wall but may show exophytic growth with irregular, cauliflower mass formation that grows into the lumen and invades the gallbladder wall. Such mass-forming invasive carcinoma may mimic ICPN macroscopically, but histologically, it is composed of overwhelmingly high-grade atypical glands showing complex papillo-tubular structures and cribriform structures, which may be associated with necrosis and even invasion within the mass (Fig. 16.2c, d).

3. Metastatic lesions

   Some metastatic tumors (e.g., malignant melanoma, renal cell carcinoma) characteristically form a polypoid mass projecting into the gallbladder lumen. Metastatic colorectal cancer may mimic intestinal-type ICPN.

#### 16.2.1.5 Prognosis

The 3-year survival rate is 90–100% for ICPN cases without invasion and 60–75% for those associated with invasion [3, 7]. The prognosis for invasive carcinoma arising in ICPNs is better than for other invasive carcinomas of the gallbladder, and this survival advantage persists even with stage-matched comparison. Death may occur in long-term follow-up even in noninvasive cases, so thorough sampling and careful evaluation are very important in these neoplasms to rule out the presence of invasive carcinoma.

## *16.2.2 Intra-Ampullary Papillary-Tubular Neoplasm (IAPN)*

### 16.2.2.1 Overview

Ohike et al. proposed a unified terminology of intra-ampullary papillary-tubular neoplasm (IAPN) for exophytic (papillary or polypoid), mass-forming preinvasive neoplasms that occur predominantly or exclusively within the ampulla of Vater [4]. IAPNs embrace all subtypes of adenomas (if they show exophytic mass formation) and noninvasive pancreatobiliary papillary neoplasms in the WHO 2010 classification [2]. IAPNs comprise about 30% of all ampullary tumors. Most patients are men (male to female = 2:1) in their 60s, who present with jaundice, occasionally accompanied by weight loss and abdominal pain. Similar to analogous tumors of the pancreas and biliary tract, these tumors also have a mixture of papillary and tubular growth, demonstrate different cellular lineages, and exhibit a spectrum of dysplasia. Approximately 80% of IAPNs involve invasive components, and 28% of invasive carcinomas within the ampulla arise in association with IAPNs [4].

### 16.2.2.2 Gross Features

IAPNs are characterized by preinvasive exophytic components that grow almost exclusively (> 75%) within the ampullary channel and/or the very distal segments of the common bile duct or pancreatic duct, with only minimal (< 25%), if any, involvement of the duodenal aspect of the papilla. IAPNs may fill the lumen of the ampullary ducts, resulting in marked dilatation of the upstream bile duct and pancreatic duct. The mass presents as a submucosal, protruded tumor on the duodenal surface by endoscopic observation and may be exposed or nonexposed from the orifice of the ampulla of Vater (Fig. 16.1d and 16.5). The mean tumor size is 2.7 cm [4].

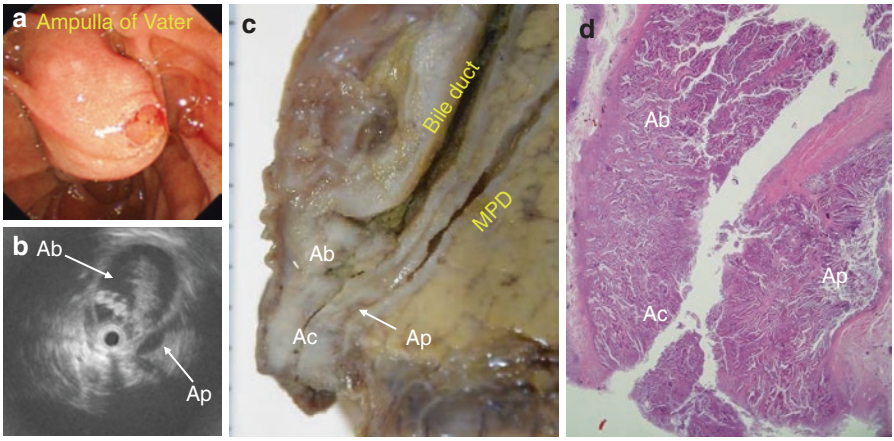


**Fig. 16.5** Endoscopic (**a, b**) and pathologic (**c, d**) features of IAPN showing a submucosal, protruded tumor due to intraductal exophytic, papillary growth within the ampulla of Vater. Ac, ampullary channel; Ab, intra-ampullary bile duct; Ap, intra-ampullary pancreatic duct; MPD, main pancreatic duct

#### 16.2.2.3 Microscopic Features

IAPNs show intraductal growth within the ampulla of Vater, involving periampullary ductules within the sphincter of Oddi. Most IAPNs have a mixture of both papillary and tubular growth (Fig. 16.4 and 16.5d); some show predominantly papillary, while others predominantly tubular. IAPNs show a spectrum of dysplastic change (may exhibit typical adenoma-carcinoma sequence) and usually contain high-grade dysplasia focally, substantially, or extensively. Half of cases show a mixture of cell lineage morphology, and IAPNs exhibit intestinal-tubular type the most often, followed by pancreatobiliary and gastric type as the predominant pattern. IAPNs with substantial or extensive high-grade dysplasia comprise more than 80% of the cases associated with invasive carcinoma. The cell lineage in the invasive component is usually the same as that of the preinvasive component, but some intestinal-type IAPNs transform into pancreatobiliary-type invasive carcinoma [4].

#### 16.2.2.4 Differential Diagnosis

Nonneoplastic polyps, intestinal-type adenomas, invasive papillary carcinomas, and metastatic lesions, which may protrude into the lumen of the ampullary ducts, must be excluded from the category of IAPN.

1. Nonneoplastic polyps/hyperplastic lesions:

   i. **Adenomyomatous hyperplasia (adenomyoma, myoepithelial hamartoma):**

   Adenomyomatous hyperplasia forms a white, firm, nodular, or pedunculated lesion of the ampulla of Vater. Its size ranges from 10 to 30 mm, which may cause long-term biliary obstruction. The histology shows multiple glandular structures surrounded by fibroblastic/myofibroblastic proliferation. The epithelial cells exhibit gastric metaplasia (MUC5AC and MUC6 positive) and low proliferative activity but lack cellular atypia. Some cases may develop from heterotopic pancreas.

   ii. **Brunner's gland hyperplasia and periductal gland hyperplasia/proliferation:**

   The localized lesions form a sessile or pedunculated lesion which may mimic gastric-pyloric-type IAPNs.

   iii. **Distended glands (overreplacement of ampullary mucosa):**

   The ampullary mucosa commonly overgrows beyond the orifice, replacing a portion of the surrounding duodenal mucosa, termed "distended glands" [8]. The lesions are localized on the Oddi's sphincter muscle extension. The epithelial cells often exhibit gastric metaplasia (MUC5AC positive) and may show low proliferative activity but lack cellular atypia. IAPNs may arise in association with distended gland elements (Fig. 16.4a).

2. Intestinal-type adenoma

   Intestinal-type adenomas, which are histologically similar to colonic adenoma, usually arise from the duodenal-type mucosa or transitional mucosa covering the ampulla and often project into the duodenal lumen as a pale, soft elevation, or plaque. Less commonly, the adenoma is confined within the ampulla, resulting in a prominent bulging ampulla covered by intact mucosa, which may be indistinguishable from intestinal IAPN except for the presence of an exophytic mass.

3. Invasive papillary adenocarcinoma

   Invasive papillary adenocarcinomas may show exophytic growth with irregular, papillary, or clumpy mass formation that grows within the ampulla. Histologically, they are composed of overwhelmingly high-grade atypical glands showing complex papillo-tubular structures and cribriform structures, which may be associated with necrosis and even invasion within the mass. In addition, they usually demonstrate expansive or infiltrative, invasive growth at the bottom of the tumors.

4. Metastatic lesions

   Pancreatic neoplasms and bile duct neoplasms of various types invade or extend into the ampulla of Vater and may produce IAPN-like mass components. Similar to the gallbladder, metastatic renal cell carcinoma and malignant melanoma may involve the ampulla of Vater and form a polypoid mass within the ampulla.

#### 16.2.2.5 Prognosis

Noninvasive IAPNs have an excellent prognosis. IAPNs with invasion exhibit malignant behavior but have a significantly better prognosis than ordinary invasive ampullary carcinomas unaccompanied by IAPNs (3-year survival rate, 69% vs. 44%) [4]. The survival advantage in patients with invasive IAPN may be attributable to the earlier clinical detection of IAPN than other non-IAPN-associated carcinomas.

## 16.3 Summary

Exophytic preinvasive neoplasms (ICPNs and IAPNs) of the gallbladder and ampulla of Vater are analogous to their pancreatic and biliary counterparts (IPMNs and IPNBs). They show variable cellular lineages, a spectrum of dysplasia, and a mixture of papillary or tubular growth patterns. ICPNs and IAPNs are relatively indolent neoplasia; even those with invasion have a significantly better prognosis than ordinary invasive carcinomas unaccompanied by ICPNs or IAPNs. ICPN/IAPN should be strictly distinguished from “invasive papillary adenocarcinoma” or “mass-forming invasive adenocarcinoma,” which are types of invasive carcinomas. These new concepts appear to be clinically and pathologically significant.

**Conflict of Interest Statement** The author has no conflicts of interest or financial ties to disclose.

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