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REVIEW

Cholangiocarcinoma: Classification, Histopathology and Molecular Carcinogenesis

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Abstract

Cholangiocarcinoma (CC) is the second most common tumor of the liver, originating from the biliary system with increasing incidence and mortality worldwide. Several new classifications review the significance of tumor localization, site of origin, proliferation and biomarkers in the intrahepatic, perihilar and distal forms of the lesion. Based on growth pattern mass-forming, periductal-infiltrating, intraductal, undefined and mixed types are differentiated. There are further subclassifications which are applied for the histological features, in particular for intrahepatic CC. Recognition of the precursors and early lesions of CC including biliary intraepithelial neoplasia (BilIN), intraductal papillary neoplasm of the bile ducts (IPNB), biliary mucinous cystic neoplasm (MCNB) and the candidate precursors, such as bile duct adenoma and von Meyenburg complex is of increasing significance. In addition to the previously used biliary markers detected by immunohistochemistry, several new markers have been added to the differentiation of both the benign and malignant lesions, which can be used to aid in the subclassification in association with the outcome of CC. Major aspects of biliary carcinogenesis have been revealed, yet, the exact way of this diverse process is still unclear. The factors contributing to molecular cholangiocarcinogenesis include various risk factors, different anatomical localizations, multiple cellular origins, genetic and epigenetic alterations, tumor microenvironment, heterogeneity and clonal evolution. Driver mutations have been identified, implying that they are optimal candidates for targeted therapy. The most promising therapeutic candidates have entered clinical trials.

Keywords Cholangiocarcinoma · Liver cancer · Biliary markers · Stem cells · MicroRNA

Introduction

Cholangiocarcinoma (CC) is a malignant tumor of the epithelium lining of the biliary tree with very poor outcome, showing an increase in incidence and mortality rate worldwide [1–4]. It is less common than hepatocellular carcinoma (HCC), however it is still the second most common primary liver cancer [5–8]. The incidence of CC is high in the Far East and much lower in the Western countries by reason of the different risk factors which are associated with ethnic, genetic and environmental predispositions [1]. Two-thirds of CCs derive from the extrahepatic bile ducts, one-third from the intrahepatic bile ducts and all cases are rarely diagnosed before the age of 40, the mean age being 50 (except for patients with primary sclerosing cholangitis), with a slight male predominance [3, 7, 9].

There are several excellent reviews that summarize the guidelines for the diagnostic criteria, prognosis, therapeutic approaches and molecular profiling of CC [1, 8, 10–12]. The heterogeneity of CC was previously recognized using a number of new expressional markers and clinical data. Several classifications were suggested based on the localization, histopathological appearance and molecular alterations detected

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in CC [6, 10, 11, 13–16], which might reflect the differences in morphology and biological characteristics of the bile duct epithelium [11, 17–19]. The anatomic classification, the mixed HCC-CC cases (less than 1% of liver cancers), the epidemiology, the relation to cirrhosis, chronic hepatitis B (HBV) and C virus (HCV) infections [20], and to primary sclerosing cholangitis (PSC), the proliferative and inflammatory gene signatures in CC etc. have all been extensively discussed recently [1, 10, 13, 21].

Since CC has a very poor outcome, much effort has been made to disclose the mechanism of cholangiocarcinogenesis and progression of CC [4, 22–28]. The studies and experimental evidences have clarified certain aspects of biliary carcinogenesis, yet the exact way of how these processes occur are still unclear. Numerous factors have been hypothesized to be involved in biliary carcinogenesis, such as stem/progenitor cells, genetic and epigenetic alterations, tumor microenvironment and exposure to carcinogenic agents. All of which may collectively contribute to the heterogeneity observed between patients and within the same tumor.

The present review is provided from a pathological point of view, with focus mainly on *classification, histopathological diagnostic criteria, precursors and early lesions, factors of molecular cholangiocarcinogenesis.*

Classification of Cholangiocarcinoma and Histopathological Diagnostic Criteria

Several guidelines and classifications exist for the management of CC [8]. The most recent classification is based on the anatomical localization of the tumor and ***intrahepatic (iCC), perihilar (pCC) and distal (dCC)*** forms have been generally accepted (Table 1) [1, 3, 10, 15, 16], which have different epidemiology, pathogenesis and clinical presentations [1] (Fig. 1). The previously used term "Klatskin tumor" for *pCC* is discouraged [1].

Intrahepatic CC can be further divided into *large bile duct* and *peripheral iCC* (Table 1), the latter arising from the small bile ducts [6, 29]. The large bile ducts contain peribiliary glands (PBG) within their walls, in contrast to the small bile ducts which are connected with the bile ductules or canals of Hering [29]. *iCC* derives from the hepatic stem/progenitor cells, which are located either in the small bile ducts and ductules/canals of Hering or in the peribiliary glands of the large bile ducts [6, 30]. This might explain why the characteristics of *iCCs* arising from different anatomical localizations vary from each other [29]. *iCC* can be further classified into mass-forming (60–80%), periductal-infiltrating (15–35%), intraductal (8–29%), undefined and mixed subtypes which are related to the prognosis (Table 2) [2, 10, 29]. (Fig. 2a–d). **Histologically**, *iCC* is an adenocarcinoma showing varying degree of differentiation from well-to-moderate and poor, with certain rare variants [5], (Table 3). *iCC* deriving from large bile ducts contains larger tubules or papillary growth with taller columnary cells (Fig. 3a,b). *iCC* of small duct type consists of cuboidal cells forming small tubular or trabecular structures.

Perihilar CC *(pCC)* and ***distal CC*** *(dCC)* have the morphology of exophytic and/or intraductal (endophytic) tumors, which might be periductal (the most common type) or nodular (Table 1). There are data suggesting that better prognosis is associated with the intraductal form [10]. **Histologically,** *pCC* and *dCC* have similar appearances as *iCC* deriving from the large bile ducts. The cells of origin of *pCC* and *dCC* have been proposed as mucin-producing cholangiocytes lining the large bile duct and/or the hepatic progenitor cells [3].

Table 1 Classification of cholangiocarcinoma (CC) based on anatomical localization *

Intrahepatic CC (iCC)
large bile duct type
small bile duct type (peripheral)
Perihilar CC (pCC)
Distal CC (dCC)

*Based on [3, 15, 29], AJCC and UICC

Immunohistochemical Typing and Biomarkers of CC

It might be a diagnostic challenge to **differentiate CC, especially *iCC* from metastasis** of the pancreas and other neoplasms with adenocarcinoma morphology with the help of immunohistochemistry (Supplementary Table 1).

More recently Fernández Moro et al. [32] established an integrative immunohistochemical classification of adenocarcinomas of the pancreatobiliary system. Cluster and differential expression analysis of an immunohistochemical panel of 38 antibodies resulted a characteristic profile for *iCC* and intestinal type adenocarcinoma that can distinguish them from metastatic and pancreatobiliary adenocarcinomas [32]. These authors proposed an immunohistochemical panel including CK19, CK20, MUC2, MUC5AC, CA19–9, mCEA, CA125 and SMAD4 for the differentiation [32]. Three subtypes, extrahepatic pancreatobiliary, intestinal and intrahepatic CC, were classified by immunohistochemistry "with differences in prognosis, biology and potentially in response to treatment" and according to the obtained results the diagnosis of *iCC* "no longer needs to be regarded as a diagnosis of exclusion" [32]. A combination of other markers (such as CK7, CK17, maspin, vimentin) might increase the diagnostic accuracy further [32]. (Fig. 4 and Supplementary Table 1).

The differentiation of *iCC* from HCC might necessitate additional markers, such as HepPar-1, Arginase-1, Glutamyl Synthetase (GS), Glypican 3, Heat Shock Protein 70 (HSP70) to help establish the diagnosis [33].

CCs show increased expression of tight junction proteins (TJ), such as claudins 1, 3, 4, 7, occludin etc. which is a

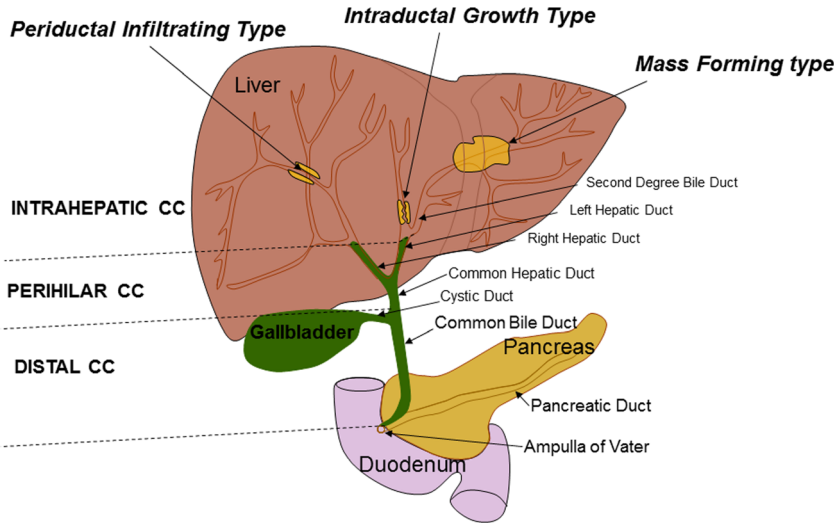

Fig. 1 Classification and growth patterns of cholangiocarcinoma (CC) based on anatomical localization

valuable tool for distinguishing CC from HCC, but not from pancreatic ductal carcinoma, which expresses similar claudin subtypes [34, 35]. By discriminant analysis, *iCC*, extrahepatic CC and gallbladder cancer could be differentiated from each other and from their normal sites of origin of the biliary tree based on TJ protein pattern [18, 19].

CC is predominantly diagnosed in an advanced clinical stage, when curative treatment is usually unsuccessful in the majority of cases and the prognosis is poor. This in part is due to the symptomless manifestation of the tumor in the early stage, especially in case of *iCC*, and in part to the lack of specific and sensitive biomarkers [21]. Several ***biochemical markers*** are used extendedly in the diagnostic panel, such as aminotransferases, total bilirubin etc. and also certain tumor markers, such as carbohydrate associated antigen 19–9 (CA19–9), CA125, carcinoembryonic antigen (CEA), however all with variable diagnostic value.

Prognostic Factors

Several factors have been studied regarding the outcome of CC. Tumor size, anatomical localization, solitary vs multiplex tumors, lymph node status, tumor stage, surgical resection margins, vascular invasion, histopathological and immunohistochemical types/subtypes [32], biomarker (CA19–9 etc) levels were found to have varying values as independent predictors of survival in addition to the age and gender of patients [2]. (Supplementary Table 1) In a recent survival analysis, extrahepatic pancreatobiliary type had the poorest overall survival in contrast to the intestinal type, which had a better prognosis [32].

Table 2 Classification of cholangiocarcinoma (CC) based on growth pattern and gross (*)

Mass-forming (60–80%): nodular, exophytic
Periductal-infiltrating (15–35%)
Intraductal (8–29%): papillary, polypoid
Undefined
Mixed

*Based on [2, 10, 29, 31]

Staging of CC

Staging systems are separately used for *iCC*, *pCC* and *dCC*, which are defined by the American Joint Committee on Cancer (AJCC), Internationale Contre le Cancer (UICC) and the Liver Cancer Study Group of Japan, and are updated regularly in new editions. This reflects that with the extension of our knowledge and the introduction of new biomarkers, better discrimination has become available in the evaluation of prognostic factors [2].

Precursors and Early Lesions of CC

It is generally accepted that CC is a multistep process originating from transformed biliary epithelial cells or from stem cells [13]. Several lesions and entities have been described as precursors for the different subtypes of CC (Table 4).

The terms ***biliary intraepithelial neoplasia*** **(BilIN)** and ***intraductal papillary neoplasm of the bile ducts*** **(IPNB)** were introduced for intrahepatic large duct, perihilar and distal CC [5, 41]. Invasive biliary adenocarcinomas were shown to be preceded by the „flat" (non-tumor-forming) BilIN or the „papillary" (tumor-forming) types [36]. Several other possible premalignant candidate lesions, such as *bile duct adenoma (peribiliary gland hamartoma), biliary fibroadenoma, von Meyenburg complex, ductal plate malformations, cystic and micropapillary epithelial changes of peribiliary glands* were recognized [6, 36, 37].

BilIN has been accepted by the World Health Organization (WHO) as a specific entity, usually recognized microscopically, and has been further divided into grades 1, −2, −3 (low-, moderate- and severe dysplasia) based on the degree of

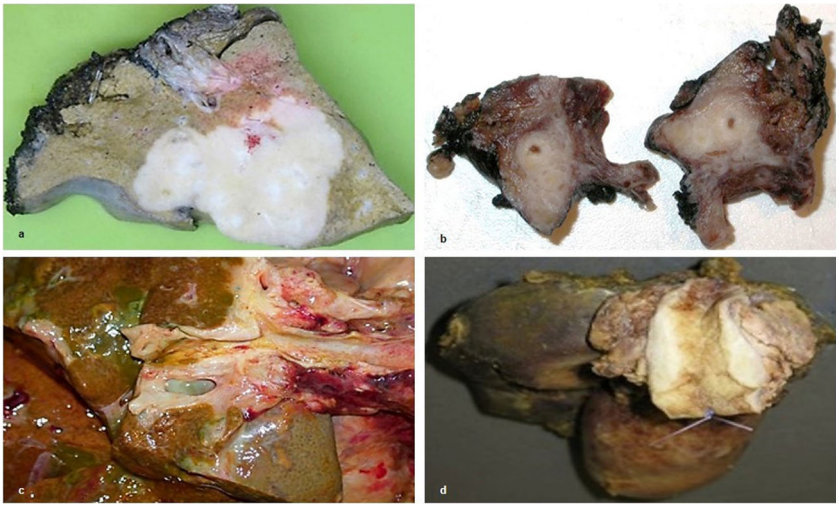

Fig. 2 Macroscopy of cholangiocarcinoma (CC). **a** Mass-forming type of intrahepatic CC, **b** periductal infiltrating type of CC (large bile duct type), **c** perihilar CC, **d** distal CC

structural differentiation and atypia (Fig. 5). It might be unrecognizable by macroscopy, or has a flat gross appearance, being less than 2–3 mm tall and can occur in the large intrahepatic or extrahepatic bile ducts and in the PBGs [41]. Microscopically, however, the „flat type" might have flat or micropapillary appearance [36, 37, 41]. The diagnostic criteria have been defined by an international consensus [42], based on the degree of cellular/nuclear atypia, nuclear pseudostratification, protruding nuclei to the apical surface and loss of cellular polarity [37]. Several authors emphasize that BilIN is a biliary counterpart of the pancreatic intraepithelial neoplasia (PanIN) in the pancreas [37, 38].

There are several molecular markers that can be detected by immunohistochemistry in BilIN lesions. Biliary cytokeratins, such as CK7 and CK19 are usually strongly expressed [37], whereas P53, p21, cyclinD1 are overexpressed [37]. Enhancer of zeste homologe 2 (EZH2) has been shown to be overexpressed in CC [43] and p16 increases parallel with the grade of BilIN [44]. From the viewpoint of diagnostics, the immunohistochemical detection of S100P was shown to be useful, being mostly negative in reactive biliary lesions and increased in BilIN2, −3 and CC.

Table 3 Classification of cholangiocarcinoma (CC) based on histological features (*)

Based on differentiation
well, moderately, poorly differentiated
Classification of intrahepatic CC (*)
Conventional type (bile duct type)
Small bile duct type (peripheral type)
Large bile duct type
Bile ductular type
Intraductal type
Combined CC/hepatocellular carcinoma
Rare variants
Squamous/adenosquamous type
Undifferentiated type
Lymphoepithelial type
Mucinous/signet ring, clear cell type
Others

*Based on [3, 31]

The preinvasive pancreatobiliary neoplasm includes the ***IPNB***, which is a macroscopically visible exophytic intraductal papillary growth with low-, intermediate- and high grades, a counterpart of the similar tumor arising in the pancreas, the so called intraductal papillary mucinous neoplasm (IPMN) [31, 39, 41, 45]. The papillary tumor fills the bile ducts leading to a fusiform dilatation sometimes associated with mucin-containing cyst formation [11, 36]. The tumor might arise in large intrahepatic or extrahepatic bile ducts as well as in PBGs. IPNBs might be multiple along the biliary tree and recurrence after surgery is common. The lesion has four histological subtypes: pancreatobiliary, intestinal, gastric and oncocytic [36, 40, 45–48], however, some tumors contain more than one subtype. MUC1 is mostly expressed in the pancreatobiliary, while MUC2 in the intestinal type [41]. MUC5AC is positive in all four types. Cytokeratin 20 is mainly positive in the intestinal type, but not in the gastric and oncocytic types [40]. High-grade IPNB or in situ carcinoma are both often associated with invasion according to Nakanuma et al. [41] at the time of surgical resection, especially those arising in the extrahepatic bile ducts. More recently IPNB has been classified into two subtypes [41, 45, 49]. The "pancreatic" (type I) is more similar to IPMN, mainly located in intrahepatic and/or hilar bile ducts with cystic dilatation. The "non-pancreatic" (type II) subtype can be detected mainly in the extrahepatic bile ducts with frequent high-grade dysplasia and is more aggressive. Some authors use the term "papillary cholangiocarcinoma" for this type of IPNB [50].

The ***bile duct adenoma*** (BDA, peribiliary gland hamartoma, intrahepatic bile duct adenoma) is a rare benign

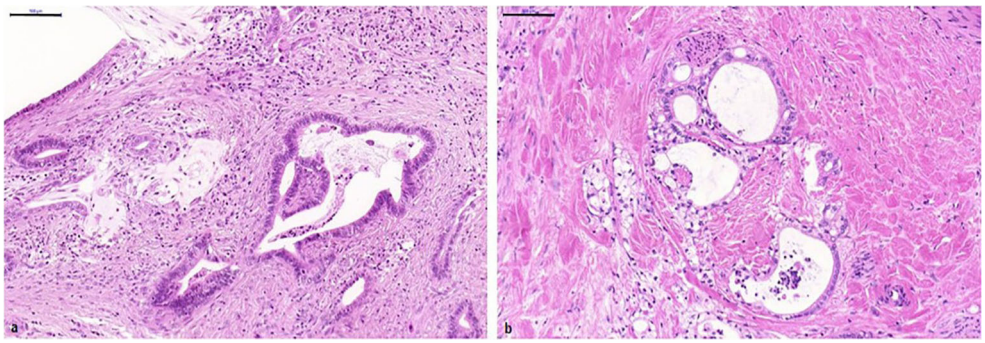

Fig. 3 Different types of intrahepatic cholangiocarcinoma. **a** large bile duct type (scale bar: 1000 μm), **b** large bile duct type (scale bar: 200 μm)

tumor, originating from the cholangiocytes, usually in subcapsular localization with a size of 1–20 mm in diameter [51, 52]. The tumor is composed of closely packed small ductules with varying degree of cellular atypia, usually no cystic dilatation and with intervening fibrotic stroma. Sometimes it might be problematic to distinguish BDA from the ductular reaction, a frequent finding in chronic liver diseases and from well differentiated CC, especially in intraoperative frozen sections [43, 52, 53]. The epithelial cells express CK7 and CK19, epithelial membrane antigen (EMA) and low proliferation activity detected by Ki67 immunoreaction [54].

Biliary adenofibroma (BAF) is a benign tumor similar to BDA with increased fibrotic stroma and glandular cystic dilatation.

The ***von Meyenburg complex*** (VMC, biliary microadenoma) is a congenital benign lesion composed of small bile ducts and ductules of varying sizes, forms and dilatations and occasionally the lesion contains bile. It is usually multiplex and as in case of BDA, is mainly subcapsular in localization (Fig. 6). There are reports, however, suggesting that the VMC is a precursor lesion for *iCC* of the peripheral subtype and is associated with *iCC* [55–57].

Cystic and Micropapillary (C-P) Epithelial Changes in Peribiliary Glands (PBGs) intramural and extramural PBGs are located along the extrahepatic and intrahepatic large bile ducts with mucin and/or serous secretion production [58]. By immunohistochemistry, C-P lesions were found to have increased mucin secretion and MUC5AC, cyclin D1 and S100P expressions as compared to cystic lesions [58]. These authors concluded that C-P lesions might be precursors of biliary neoplasms, especially of intraductal papillary neoplasm and mucin-producing CC [58]. Multipotent stem/progenitor cells were detected in PBGs, which have been suggested to participate in the carcinogenesis [30, 59, 60].

Molecular Cholangiocarcinogenesis

Several papers report that molecular cholangiocarcinogenesis is a complex multifactorial process, in which risk factors [24], persistent inflammation of the bile duct epithelium, genetic and epigenetic alterations, multicellular origin and tumor heterogeneity are involved [22, 61–63]. According to the classical belief, cholangiocarcinogenesis is promoted

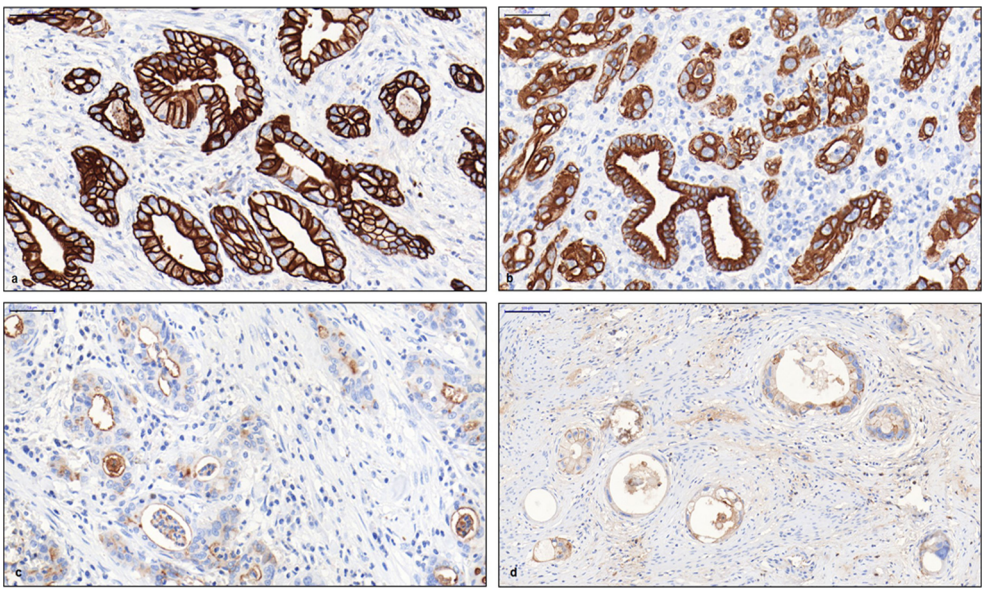

Fig. 4 Positive immunostaining for citokeratin7 (CK7), CK19, MUC1 and tight junction protein Claudin-4 (scale bars: 50 μm)

Table 4 Precursors and early lesions of cholangiocarcinoma

Biliary intraepithelial neoplasia (BilIN) [6, 11, 36–39]
BilIN-1, BilIN-2, BilIN-3
Intraductal papillary neoplasms of the bile ducts (IPNB) [11, 36, 37, 39, 40]
Intraductal tubular (tubulopapillary) neoplasms of the bile ducts (ITPN-B)
low-, high-grade lesions
pancreatobiliary, intestinal, oncocytic, gastric phenotype
Biliary mucinous cystic neoplasms (MCNB)
Bile duct adenoma (BDA) (peribiliary gland hamartoma), biliary adenofibroma* [6]
von Meyenburg complexes (VMCs) (biliary microhamartoma)* [6]
*"candidate precursor" for peripheral CC

Based on [6, 11, 36–40]

by dysregulated reparative proliferation of cholangiocytes, which leads to DNA damage, overproduction of mitogenic factors (IL-6, TGF-β) and overactivation of pro-proliferative intracellular signaling pathways (TGF-β, AKT, mTOR, MAPK, WNT, Hedgehog), and subsequently to activating mutations (KRAS, BRAF), overexpressed and silenced genes (EGFR, HGF and TP53, p16, respectively) [28, 61, 64].

Based on molecular alterations, biliary tract cancer-related biomarkers have also been suggested, e.g. Erb/HER family (Erb-1/EGFR, Erb-2/HER-2, ErbB/HER-3), vascular endothelial growth factor (VEGF), mitogen-activated protein kinase (MAPK), PIK/PTEN/AKT/mTOR and several other biomarkers in serum and bile, such as CA19–9, matrix metalloproteinases (MMPs), serum CK19 fragment (CYFRA 21-1), SMAD family member 4, isocitrate dehydrogenase (IDHs) and microRNAs [21]. More recently, specific genomic profiling has provided new possibilities in the detection of CCs opening the path for targeted therapy [4, 13, 14, 65]. Nevertheless, no diagnostic biomarkers have been found to screen for

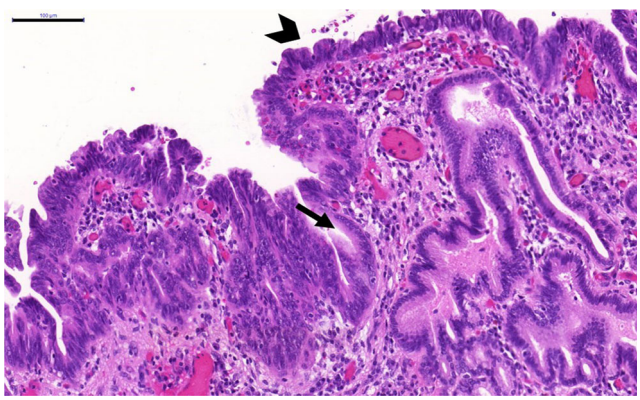

Fig. 5 Biliary intraepithelial neoplasia (BilIN) (arrow: BilIN 1, arrowhead: BilIN 2; scale bar: 100 μm)

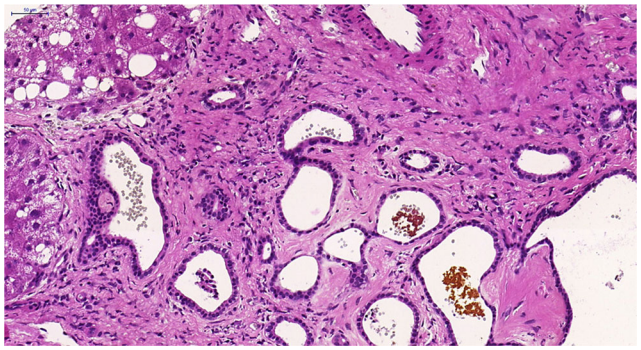

Fig. 6 Dilated and angulated bile ducts in fibrous stroma in von Meyenburg complex (scale bar: 50 μm)

patients at higher risk of CC and to identify the disease at an early stage or pre-cancerous phase [13, 66].

Nevertheless, genome and exome sequencing studies have identified the predominant molecular alterations in CC [10, 13] and in early lesions of CC [67].

Cholangiocarcinoma-Related Genetic Alterations

In recent years, a great amount of effort has been put in to reveal the CC-associated genetic alterations and it turned out that numerous genes are affected by recurrent somatic alterations not only in CCs but in BilIN and IPNB, as well [9, 61, 68]. For example, KRAS mutations (codon 12) have been observed in 33% of BilIN and about 40% of IPNB lesions [61, 69] and in patients with hepatolithiasis, KRAS mutation is regarded as an early molecular event during the progression of iCC [70].

In CC, diverse types of genetic alterations (e.g. mutation, amplification, deletion, fusion and methylation) have been described [71]. These alterations affect various genes involved in different cellular processes ranging from cell cycle control, cytokine signaling, genomic stability and chromatin remodeling to DNA repair [12–14, 61, 65, 72–74] (Table 5). It has been observed that the frequencies of the genetic alterations varies in association with anatomic location (iCC, eCC) and etiologic exposure (e.g. liver fluke or non-liver fluke CC types) [9, 84, 87]. According to the summarized mutation frequencies published in the various articles, FGFR, IDH1, IDH2, BAP1, PBRM1, MCL1, CDKN2A, BRAF and BRCA1/2 genes seem to be more frequently altered in iCC, while TP53, KRAS, CDKN2B, SMAD4, ErbB2/HER2, CTNNB1 and MLH1 gene alterations are more frequent in eCC (Table 5). Similar data has been published in a handful of previous summaries [9, 14, 62, 65, 75, 84, 88, 89]. In addition, some of these mutations seem to be co-occuring, while others are mutually exclusive [61, 76, 84, 90].

Table 5 Number of patients found to harbor the actual genetic alteration in relation to the total number of examined iCC and eCC cases

Gene group	iCC	%	eCC	%	Reference
Genomic stability:					
TP53	189/779*	24.3	110/298	36.9	[4, 71, 75–83]
STK11	4/178	2.2	3/105	2.9	[77, 79–81]
MAPK signaling:					
KRAS	193/1108	17.4	117/372	31.5	[4, 71, 75–86]
NRAS	9/172	5.2	14/203	6.9	[75–79, 81, 82]
BRAF	32/672	4.8	3/170	1.8	[4, 77, 79, 80, 82, 85]
ErbB2/HER2	15/582	2.6	22/252	8.7	[4, 71, 75–77, 80]
ErbB3	2/28	7.1	–	–	[78]
ErbB4	2/105	1.9	0/95	–	[77, 79]
AKT/PI3K signaling:					
EGFR	8/356	2.2	2/131	1.5	[77, 79, 84, 85]
EGFR amplification	5/482	1.0	0/114	–	[4, 77]
MET	10/510	2.0	0/114	–	[4, 77, 78]
PI3KCA	38/724	5.2	11/171	6.4	[4, 76–81, 83]
PIK3C2A	7/137	5.1	5/95	5.3	[77, 79, 81]
PTEN	14/308	4.5	10/204	4.9	[75, 77–81, 83]
Angiogenesis:					
VEGFA	1/32	3.1	–	–	[81]
KDR	5/252	2.0	6/114	5.3	[76, 77, 79, 80, 83]
WNT signaling:					
WT1	1/28	3.6	–	–	[78]
CTNNB1	0/109	–	3/104	2.9	[76, 77, 79]
Cell cycle control:					
CDK6	2/28	7.1	–	–	[78]
CDKN2B	1/28	3.6	15/99	15.2	[75, 78]
CDKN2A	131/622	21.1	42/270	15.6	[4, 75–81]
CCND1	4/45	8.9	7/118	5.9	[75, 76, 80]
TGF-β signaling:					
SMAD4	7/266	2.6	32/223	14.3	[71, 75–77, 79, 83]
TGFBR2	5/137	3.6	6/95	6.3	[77, 79, 81]
DNA repair:					
MLH1	0/105	–	2/95	2.1	[77, 79]
MSH2	1/28	3.6	–	–	[78]
MSH6	1/28	3.6	–	–	[78]
BRCA1/2	4/69	5.8	–	–	[78, 80]
Epigenetic regulation:					
IDH1	163/1111	14.7	2/265	0.8	[4, 71, 76–86]
IDH2	17/320	5.3	1/105	0.9	[77–81, 85]
ARID1A	131/853	15.4	36/281	12.8	[4, 71, 75, 77–81, 83, 86]
BAP1	129/888	14.5	11/256	4.3	[4, 71, 77–81, 84, 86]
PBRM1	54/448	12.1	15/298	5.0	[71, 75, 77, 79–81, 84, 86]
MCL1	6/28	21.4	–	–	[78]
FGFR fusions:					
	121/764	15.8	0/141	–	[4, 78, 80, 81, 84, 85]

iCC: intrahepatic cholangiocarcinoma; eCC: extrahepatic cholangiocarcinoma

* Cases with mutation/total examined cases

Deep-sequencing studies also disclosed that the average genetic alteration per patient ranges from 1.3 to 3.6 in iCC and from 1.18 to 4.4 in eCC and the majority of CCs have at least one driver gene mutation [4, 71, 77–79]. In 5–22% of CCs, however, none of these main genetic alterations could be found [71, 77].

Additionally, methylation [91], mismatch repair gene-deficiency [78] copy number alterations [89, 92, 93] and microsatellite instability [94] have also been observed in CC. Genes frequently inactivated by methylation in CC may include MLH1, p16INK4/CDKN2A, RASSF1A, RUNX3, DAPK and SOCS-3 [91]. Furthermore, an intriguing DNA methylation assay analysis of bile fluid has identified a panel of 5 genes (CCND2, CDH13, GRIN2B, RUNX3, TWIST1) that detected CC cells with a sensitivity of 83% and a specificity of 100% [72].

Association between genetic alterations and patients' outcome has also been revealed. For example, KRAS, BRAF mutations, overexpression of EGFR, HER2, activation of oncogenic signaling pathways, DNA amplifications and deletions have been found to be associated with poor outcome [95, 96]; whereas iCCs characterized by activation of inflammatory signaling pathways, overexpression of cytokines and activation of STAT3 showed well differentiated cells and good survival [96].

Multicellular Origin of iCC

Histologic evidences, diverse cellular phenotypes and cell markers suggest that iCC cannot be originated only from transformed cholangiocytes [77, 89, 97] but from multiple types of cells, including (1) cholangiocytes or intrahepatic biliary epithelial cells (IBEC) in the large bile ducts, small intrahepatic bile ducts and PBGs, (2) hepatic progenitor cells (HPC) occurring in the canals of Hering, biliary tree stem/progenitor cells (BTSC) residing within the medium-large intrahepatic and extrahepatic bile ducts, and (3) mature hepatocytes [25, 61, 89, 98]. In murine experiments, transformed hepatocytes, hepatoblasts, and HPCs were capable of producing a broad spectrum of liver malignancies ranging from CC to HCC, which may occur due to cellular plasticity of liver cells [89, 98–100]. There are key pathways promoting and maintaining progenitor cell-like traits, such as Notch, Wnt/β-catenin, Hedgehog, Hippo, PI3K/AKT/PTEN, IL-6/TGF-β and RAS/RAF/MEK/ERK, providing feasible targets for cancer therapy [26]. Additionally, a whole-exome sequencing study has revealed that similar substitution mutations are predominant in iCC and HCC [23].

Combined HCC-CC showing clinicopathological, morphological, and genetic characteristics typical of both HCC and iCC also suggests that HCC and iCC share a common cellular origin and may derive directly from HPC activation and differentiation [25]. The stem-cell features type mixed-iCC and CLC derive from the most peripheral biliary branches containing HPCs, with tumor cells showing intermediate histological features between hepatocytes and cholangiocytes [25, 27]. Nevertheless, the molecular analysis indicates that CLC is rather a distinct entity with a biliary molecular profile, low chromosomal instability, enrichment of TGF-β and immune-related signaling, which proposes a biliary committed precursor [27]. Thus, combined HCC-CC and stem-cell type mixed HCC-CC tumors seem to have a biphenotypic progenitor-like precursor but CLC stands alone as an independent biliary-derived entity, not sharing any of the molecular traits of HCC.

Alternatively, genetic mutations arising in mature cells may reprogram differentiated liver cells toward a progenitor-like state. For example, mutant IDH in genetically engineered mouse models have been found to inhibit hepatocyte differentiation causing the expansion of HPCs, while combined IDH and KRAS mutations have led to the development of premalignant biliary lesions and subsequent progression to iCC [89].

Mature hepatocytes can act as an alternative to HPCs capable of replacing their own cell population and giving rise to biliary lineage cells [101]. Kupffer cells seem to be also involved since, by differentially responding to hepatic injury, Kupffer cells may induce Notch in hepatocytes and Wnt in biliary lineage cells. The transdifferentiation of mature hepatocytes into iCC has been proven in mice by Notch-mediated conversion of the hepatocytes [61, 98]. In addition, aberrant Notch has been found to be present in 60% of iCCs [89].

Heterogeneity in CC

Tumor heterogeneity has been an important issue in tumor biology for many years as it affects patient outcome, treatment response and is needed to be considered for tumor profiling, as well [25, 102]. Accumulation of unrepaired stochastic mutations and randomly occuring genomic instability are particularly relevant in CC as the diagnosis is often made late when large macroscopic lesions have already developed and tumor cells have therefore undergone many cell divisions [103].

Both intertumoral (existing between patients) and intratumoral heterogeneity has been observed in CC [62]. A distinct heterogeneity has been associated with CC identifying for example 10% unique mutations between central and peripheral samples [104]. Recently, a high degree of spatial intratumoral heterogeneity (60.3%) has been described in iCC, which is higher than in other cancers (e.g. 39% in HCC) [23]. Further analysis revealed that 42.8% of the mutations were identified in the subclones with an average of 24.7 driver mutations. In the founder cells, in contrast, only 4.3 driver mutations could be observed and only a handful of

mutations was found to be clonal in all regions of the tumors. Additionally, genome doubling occurred before subclonal diversification but after acquisition of most of the early driver mutations [23].

Nevertheless, there are other factors that may play roles in the origin of CC-related heterogeneity. For example, multiple risk factors associated with CC, which are believed to be the largest among all human malignancies [103], considering both the established and putative risk factors ranging from biliary tract inflammation through hepatitis and cirrhosis to obesity and toxins. The diverse genetic alterations observed in association with different underlying liver diseases provide a support for this [65, 105].

Further contributing factors are the genetically distinct stem cell niches (HPCs and BTSCs) observed along the biliary tree, as these stem cells possess different susceptibility to risk factors [103, 106]. Tumor microenvironment and cancer treatment are also driving factors of heterogeneity as both may influence the architecture and genetic profile of the subclones, promoting selective growth with a survival advantage to certain subclones and eradicating those with less favorable survival advantage [26].

Cholangiocarcinogenesis-Related miRNAs

Dysregulation of microRNAs (miRNA) has been observed in cholangiopathies [25, 72, 107–110] and miRNAs are detectable not only in tissues but also in plasma and serum, which renders an easier access to miRNAs [111]. The most important cholangiocarcinogenesis-related miRNAs are summarized in Supplementary Fig. 1. The miRNAs showing dysregulation in CC are those that are mainly involved in the regulation of proliferation, tumor growth, cell cycle, angiogenesis, apoptosis, EMT, inflammation, DNA methylation and chemoresistance, suggesting that these miRNAs are linked to cholangiocarcinogenesis.

Targeted Therapy

For patients with advanced-stage or unresectable cholangiocarcinoma, the available systemic therapy includes gemcitabine and cisplatin treatment with less than 1 year median overall survival [22]. Biliary tract cancers are very heterogeneous tumors with respect to localization, pathological subtypes and various genetic defects, posing a challenge for effective treatment options. The identified driver mutations are believed to be optimal candidates for personalized targeted therapy and it has been suggested that 68% of the CC cases may have potentially drug actionable pathways [77].

The candidate molecules showing preclinical effectivity have been tested in clinical studies. The most promising therapeutic candidates include tyrosine-kinase inhibitors for FGFR fusions, small-molecule inhibitors targeting chromatin-remodelling proteins such as mutant IDH1/2, HDAC, DNMT, and the ones targeting activating mutation of ERBB2/Her-2 protein kinase [22, 28]. Other mutations having a good possibility for targeted therapy may include CDKN2A/B, molecules downstream of KRAS (MEK, AKT, mTOR), MET/HGF, MCL-1, PRKACA/B and VEGFR [28, 63]. Targeting tumor stroma (apoptotic priming) and immuntherpay are also attractive [28]. Owing to the extensive inter- and intratumoral heterogeneity of CC, multiagent therapy has been foreseen as well.

In conclusion, the extensive use of imaging and endoscopic techniques has discovered lesions in the biliary system, which might be challenging in the differential diagnosis, but has prognostic and therapeutic significance. The main questions are usually the benign or malignant, the primary or metastatic nature of the lesion, and the exact site of the tissue of origin. The diagnosis should not be regarded as a “diagnosis of exclusion” in the adenocarcinomas of pancreatobiliary tumors [32]. This might not be easy due to the similarities in the histopathology, even in the immunohistochemistry of tumors of the pancreatobiliary system. To establish the correct diagnosis it is important to analyse the clinical data in addition to the extended histopathological evaluation and further molecular characterization of the lesions is also needed.

Many factors have been revealed to contribute to the molecular character of the developed CC, including several risk factors, different anatomical localizations, multiple cellular origins, genetic and epigenetic alterations, tumor microenvironment. These factors may also contribute to clinical and pathobiological heterogeneity, which influences tumor biology, drug responses and patient outcome and poses a challenge for targeted therapy. Two-thirds of patients may harbor genomic alterations that may be addressed using personalized targeted therapies [25]. Silenced genes established by different mechanisms seem to be a frequent event in CC. The mutated genes show both individual and subclonal variances and only a few mutations seem to be clonal in a tumor. Determining the types of genetic alterations in a sample is important in order to select an effective (combination) therapy [63, 77, 78, 88]. Nevertheless, the fact that certain CC cases fail to show any genetic alterations indicate that mechanisms different from intragenic mutations (amplifications, deletions, translocations, and epigenetic anomalies) may also be responsible for cholangiocarcinogenesis [77]. For example, the systemic evolutionary theory, according to which a systemic change at cellular level and a disharmony in management of energy and waste is the prime cause of neoplastic transformation and cancer, causing the gradual dedifferentiation of a differentiated but energy-restricted cell [112].

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publication of this article.

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